INFLAMMATORY AND COAGULATION DISTURBANCES IN ACUTE PANCREATITIS

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Academic dissertation

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To my Friends

ABSTRACT

Acute pancreatitis (AP), a common cause of acute abdominal pain, is usually a mild, self-limited disease. However, some 20-30% of patients develop a severe disease manifested by pancreatic necrosis, abscesses or pseudocysts, and/or extrapancreatic complications, such as vital organ failure (OF). Patients with AP develop systemic inflammation, which is considered to play a role in the pathogenesis of multiple organ failure (MOF). OF mimics the condition seen in patients with sepsis, which is characterized by an overwhelming production of inflammation mediators, activation of the complement system and systemic activation of coagulation, as well as the development of disseminated intravascular coagulation (DIC) syndrome. In systemic inflammation, an excessive proinflammatory burst is rapidly followed by an anti-inflammatory reaction that may result in immune suppression. Similarly, rapid activation of coagulation may turn into global or selected exhaustion of physiological anticoagulant systems. Vital OF is the major cause of mortality in AP, along with infectious complications. About half of the deaths occur within the first week of hospitalization and thus, early identification of patients likely to develop OF is important.

The aim of the present study was to investigate inflammatory and coagulation disturbances in AP and to find inflammatory and coagulation markers for predicting severe AP, and development of OF and fatal outcome.

This clinical study consists of four parts. All of patients studied had AP when admitted to Helsinki University Central Hospital. In the first study, 31 patients with severe AP were investigated. Their plasma levels of protein C (PC) and activated protein C (APC), and monocyte HLA-DR expression were studied during the treatment period in the intensive care unit; 13 of these patients developed OF. In the second study, the serum levels of complement regulator protein CD59 were studied in 39 patients during the first week of hospitalization; 12 of them developed OF. In the third study, 165 patients were investigated; their plasma levels of soluble form of the receptor for advanced glycation end products (sRAGE) and high mobility group box 1 (HMGB1) protein were studied during the first 12 days of hospitalization; 38 developed OF. In the fourth study, 33 patients were studied on admission to hospital for plasma levels of prothrombin fragment F1+2 and tissue factor pathway inhibitor (TFPI), and thrombin formation capacity by calibrated automated thrombogram (CAT); 9 of them developed OF.

Our results showed significant PC deficiency and decreased APC generation in patients with severe AP. The PC pathway defects seemed to be associated with the development of OF. In patients who developed OF, the levels of serum CD59 and plasma sRAGE, but not of HMGB1, were significantly higher than in patients who recovered without OF. The high CD59 levels on admission to the hospital seemed to be predictive for severe AP and OF. The median of the highest sRAGE levels was significantly higher in non-survivors than in survivors. The *in vivo* thrombin generation was estimated by means of F1+2 levels, and no significant difference between the patient groups was found in these levels. The thrombograms of all patients were disturbed in their shape, and in 11 patients the exogenous tissue factor (TF) did not trigger thrombin generation at all ('flat curve'). All of the patients that died displayed a flat curve. Free TFPI levels and free/total TFPI ratios were significantly higher in patients with a flat curve than in the others, and these levels were also significantly higher in non-survivors than in survivors. The flat curve in combination with free TFPI seemed to be predictive for a fatal outcome in AP.

In conclusion, a significant PC pathway pathology was demonstrated in severe AP, and these defects were more frequent in patients who developed OF. Failure of TF-initiated thrombin generation in the thrombogram assay, explained by high levels of circulating free TFPI, was associated with OF and mortality in AP. Increased serum and plasma levels of CD59 and sRAGE were associated with severe AP with OF, and increased CD59 levels seemed to be predictive for severe AP and OF.

CONTENTS

ORIGINAL PUBLICATIONS

The following original publications are referred to in the text by their Roman numerals.

I. Lindström O, Kylänpää L, Mentula P, Puolakkainen P, Kemppainen E, Haapiainen R, Fernandez JA, Griffin JH, Repo H, Petäjä J. Upregulated but insufficient generation of activated protein C is associated with development of multiorgan failure in severe acute pancreatitis. Critical Care 2006; 10:R16.

II. Lindström O, Jarva H, Meri S, Mentula P, Puolakkainen P, Kemppainen E, Haapiainen R, Repo H, Kylänpää L. Elevated levels of the complement regulator protein CD59 in severe acute pancreatitis. Scandinavian Journal of Gastroenterology 2008; 43:350-355.

III. Lindström O, Tukiainen E, Kylänpää L, Mentula P, Rouhiainen A, Puolakkainen P, Rauvala H, Repo H. Circulating levels of a soluble form of receptor for advanced glycation end products and high-mobility group box chromosomal protein 1 in patients with acute pancreatitis. Pancreas 2009; 38:e215-e220.

IV. Lindström O, Tukiainen E, Kylänpää L, Mentula P, Puolakkainen P, Wartiovaara-Kautto U, Repo H, Petäjä J. Thrombin generation in vitro and in vivo in patients with acute pancreatitis: Disturbed tissue factor regulation associates with organ failure and predicts fatal outcome in acute pancreatitis. Submitted.

ABBREVIATIONS

AGE	advanced glycation end product
AP	acute pancreatitis
APACHE II	acute physiology and chronic health evaluation
APC	activated protein C
aPTT	activated partial thromboplastin time
ARDS	adult respiratory distress syndrome
AT	antithrombin
CARS	compensatory anti-inflammatory response syndrome
CAT	calibrated automated thrombography
C1INH	C1 inhibitor
CPN	carboxypeptidase N
CR	complement receptor
CRP	C-reactive protein
CT	computed tomography
CTBS	lysosomal hydrolase cathepsin B
DAF	decay accelerating factor
DIC	disseminated intravascular coagulation
EPCR	endothelial protein C receptor
ERCP	endoscopic retrograde cholangiopancreaticography
esRAGE	endogenous secretory receptor for advanced glycation end product
ETP	endogenous thrombin potential
GAG	glycosaminoglycan
GPI	glycosyl phosphatidylinositol
GRO-alpha/ CINC	growth-related oncogene-alpha/ cytokine-induced neutrophil chemoattractant
HAPS	harmless acute pancreatitis score
HMGB1	high mobility group box 1
ICAM-1	intercellular adhesion molecule 1
IL	interleukin
IL-1ra	interleukin 1 receptor antagonist
IQR	interquartile range
ISTH	International Society on Thrombosis and Haemostasis
JAM	junctional adhesion molecule

MAC	membrane-attack complex
MAPK	mitogen-activated protein kinase
MASP	mannose-binding lectin-associated serine protease
MBL	mannose-binding lectin
MCP	membrane cofactor protein
MCP-1	monocyte chemoattractant protein 1
MOF	multiple organ failure
MODS	multiple organ dysfunction score or multiple organ dysfunction syndrome
NEP	neutral endopeptidase
NF-KB	nuclear factor kappa B
NO	nitric oxide
OF	organ failure
PAF	platelet-activating factor
PAI-1	plasminogen activator inhibitor 1
PAR	protease-activated receptor
PC	protein C
PCT	procalcitonin
PECAM-1	platelet endothelial cell adhesion molecule 1
PIPL-C/D	phosphatidylinositol-specific phospholipase C/D
PLA2	phospholipase A_2
PMN	polymorphonuclear neutrophils
PS	protein S
PSTI	pancreatic secretory trypsin inhibitor
PT	prothrombin time
RAGE	receptor for advanced glycation end products
ROS	reactive oxygen species
rTFPI	recombinant tissue factor pathway inhibitor
SIRS	systemic inflammatory response syndrome
SMRP	secretin-stimulated magnetic resonance pancreatography
SOFA	sequential organ failure assessment
sRAGE	soluble form of receptor for advanced glycation end products
sTNFR	soluble tumor necrosis factor receptor
TAFI	thrombin-activatable fibrinolysis inhibitor
TAP	trypsinogen-activation peptide
TF	tissue factor
TFPI	tissue factor pathway inhibitor

TLR	toll-like receptor
TM	thrombomodulin
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
t-PA	tissue-type plasminogen activator
TPN	total parenteral nutrition
u-PA	urokinase-type plasminogen activator
VCAM-1	vascular cell adhesion molecule 1

1 INTRODUCTION

Acute pancreatitis (AP) is a common cause of abdominal pain. Its annual incidence is increasing, and is currently high in Finland; 102 episodes per 100,000 inhabitants (Pelli et al 2009). The clinical diagnosis of AP is based on characteristic epigastric pain and nausea or vomiting, combined with elevated serum levels of amylase (>3 times the upper reference limit) and/or typical AP imaging findings on computed tomography (CT) (Banks et al 2006). The most common etiology of AP is alcohol abuse or gallstones, in about 70-80% of all cases (Forsmark et al 2007). AP is usually a mild, self-limited disease with a low mortality rate. However, about 20% of AP cases are severe, with a high mortality rate of 10-25% (Swaroop et al 2004). According to the Atlanta classification, severe AP is defined by the presence of local complications (necrosis, pseudocysts or abscesses) and/or organ failure (OF) (shock, pulmonary insufficiency and renal failure) (Bradley 1993).

Multi-factorial scoring systems, based on clinical and laboratory findings, have been developed for assessing the severity of AP. These are: Ranson's score (Ranson et al 1974), Glasgow/Imrie score (Blamey et al 1984) and the Acute Physiology and Chronic Health Evaluation II (APACHE II) score (Knaus et al 1985)). For predicting mortality, organ dysfunction scores (Multiple Organ Dysfunction Score (MODS) (Marshall et al 1995) and the Sequential Organ Failure Assessment (SOFA) score (Vincent et al 1998)) have been used. These scoring systems are nevertheless often too complex to use in clinical practice.

There is no specific treatment for AP, and currently the therapy is mainly supportive. This includes adequate fluid resuscitation, monitoring for hypoxemia, and pain relief. Nearly half of the patients with severe AP have OF and therefore require management in the intensive care unit (Swaroop et al 2004). OF appears to be the most important factor leading to morbidity and mortality in AP (Banks et al 2006). Early recognition and assessment of the severity of AP are thus important, so that aggressive treatment can be started. Despite numerous attempts to find accurate predictive laboratory and imaging parameters or scoring systems (Tenner 2004; Banks et al 2006), these still present a challenge for clinicians.

The exact pathogenesis of AP is only partly known, but the key event is the intra-acinar activation of trypsinogen-inducing autodigestion of the pancreas and intrapancreatic inflammation. This can progress to systemic inflammation and cause multiorgan failure (MOF) (Banks et al 2006). Coagulation disorders (mainly consumptive coagulopathy and hyperfibrinolysis (Lasson and Ohlsson 1986a; Lasson and Ohlsson 1986b)) are known to occur in severe AP; they are related to the severity and to organ dysfunction. In the

pathogenesis of pancreatic necrosis, the pancreatic perfusion and hypoxia seem to play an important role. There is increasing evidence that microvascular disturbances (vasoconstriction, shunting, increased permeability, inadequate perfusion, and increased blood viscosity and coagulation) are significant events in the progression of AP, and that reduced tissue blood flow in AP is related to severity. (Cuthbertson and Christophi 2006)

The present clinical study investigates inflammatory and coagulation disturbances in AP. At the same time, we wanted to study whether these disturbances are associated with the severity of AP, the development of OF, and the outcome of the patients, and also, whether we could find any predictive inflammatory or coagulation markers.

2 REVIEW OF THE LITERATURE

2.1 Clinical manifestations and classification

AP is a common clinical condition (approximately 2-5% of the cases of acute abdominal disorders (Leppaniemi and Haapiainen 2006)) and its onset is usually acute. The main symptoms are epigastric pain; usually radiating to the back; nausea, vomiting, fever and tachycardia. All patients may not experience pain, however, and it has been noted that in 30-40% of patients the diagnosis of AP has only been made at autopsy (Forsmark et al 2007). Physical signs of severe disease such as ecchymoses in the flank (Gray-Turner's sign) or in the periumbilical region (Cullen sign) occurs in less than 3% of patients, and have been associated with a mortality of 37% (Meyers et al 1989). The clinical suspicion of AP is supported by the finding of an elevated serum amylase level.

AP is associated with significant morbidity and mortality. Today, the overall mortality rate of AP is about 2-5% (**Figure 1**) (Russo et al 2004; Pandol et al 2007). The mortality rate is higher in patients with necrotizing AP (approximately 17%) compared to those with interstitial AP (3%) (Pandol et al 2007).

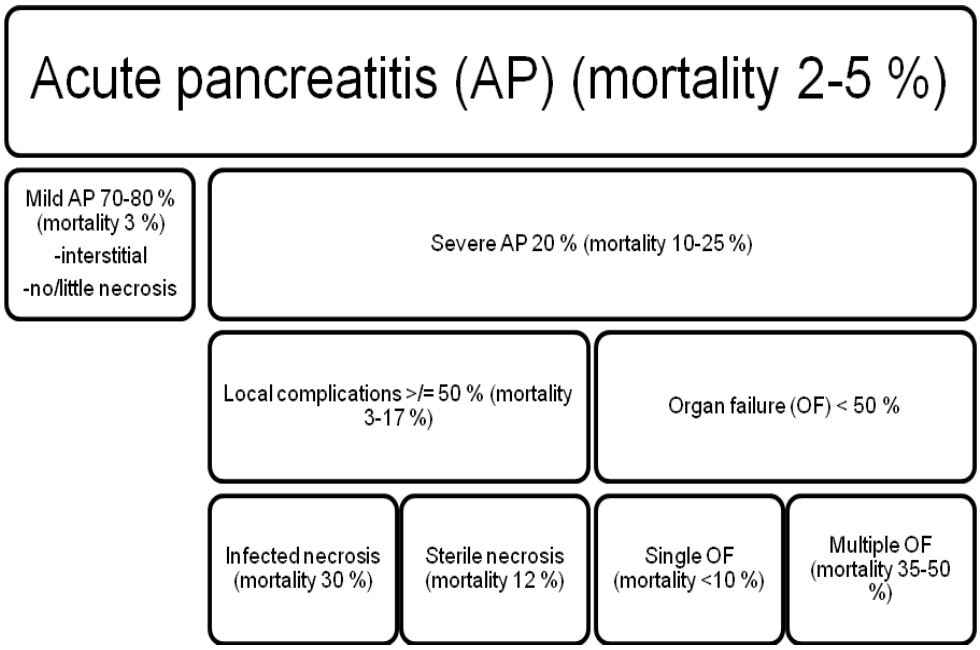

Figure 1. Acute pancreatitis, classification and mortality rates.

A widely accepted and used clinical classification system for AP was completed in an international symposium held in Atlanta, Georgia, in September 1992 (Bradley 1993). According to the Atlanta classification AP is divided into a mild and a severe disease form. The majority of cases (70-80%) are mild. Mild AP is characterized by inflammation and edema of the pancreas, interstitial pancreatitis (focal or diffuse enlargement of the pancreas) with no or little necrosis. It is usually a self-limiting disease and does not require any special treatment.

About 20% of AP cases are severe, with a rather high mortality rate, 10-25% (Isenmann et al 2001; Swaroop et al 2004). According to the Atlanta classification, severe AP is defined by the presence of local complications (necrosis, pseudocysts or abscesses) and/or OF (shock, pulmonary insufficiency and renal failure) (Bradley 1993). The mortality rate of patients with necrotizing AP is greater (ca 30%) in those with infected necrosis than in those with sterile necrosis (12%), and the prevalence of infected necrosis is approximately 15-20% (Pandol et al 2007).

Nearly half of the patients with severe AP have OF, and thus require management in the intensive care unit (Swaroop et al 2004; Banks et al 2006). The prevalence of OF is the same or somewhat higher in infected necrosis (34-89%) than in sterile necrosis (45-73%) (Banks et al 2006). In the Atlanta classification, the definition of OF includes shock (systolic blood pressure <90 mmHg), pulmonary insufficiency (PaO_2 <60 mmHg), renal failure (serum creatinine level >2 mg/dl), and gastrointestinal bleeding (>500 ml blood loss within 24 h) (Bradley 1993). OF appears to be the most important factor leading to morbidity and mortality (Banks et al 2006). In the presence of single OF, mortality is generally less than 10%, whereas in MOF the mortality rate is 35-50% (Pandol et al 2007). The first sign of MOF is often impaired lung function due to adult respiratory distress syndrome (ARDS) (Bhatia et al 2000). If OF is present already at admission to the hospital (early OF), the mortality rate is high (42%) and progresses to MOF in 79% of the patients (Isenmann et al 2001). In early OF, the mortality rate of patients with persistent OF (lasting >48 h) is 35%, but if the OF is transient (<48 h), or in severe AP without OF, the mortality rate is similar to that in mild AP (approximately 3%) (Johnson and Abu-Hilal 2004). About one half of the patients who die from AP, die within the first 1-2 weeks from a severe initial attack due to systemic inflammatory response syndrome (SIRS) and MOF. Patients who have a severe attack but survive beyond this period later develop infection complications, e.g. necrotic tissue infection leading to sepsis and SIRS and MOF. (McKay et al 1999; Pandol et al 2007)

Significant advances have been made in understanding the mechanism of action in OF since the Atlanta classification was published. It is now clear that this classification does not distinguish between the severity or reversible nature of the OF. The Atlanta classification has been criticized, and several authors have emphasized that the criteria should be revised (Vege and Chiari 2005; Bollen et al 2007; Rau 2007; Bollen et al 2008).

2.2 Epidemiology of acute pancreatitis

There are very few epidemiological studies on large patient populations with AP. Comparing the results of different reports is difficult, because some studies report the incidence of only the first attacks of AP, while others include also recurrent attacks. The annual incidence of AP has been found to be on the rise (Corfield et al 1985; Jaakkola and Nordback 1993; McKay et al 1999; Lindkvist et al 2004; Frey et al 2006; Yadav and Lowenfels 2006; Sandzen et al 2009), particularly in gallstone AP (Yadav and Lowenfels 2006), and to vary considerable in different countries. It is reported in the UK to be 7.3-31.8 (Corfield et al 1985; Thomson et al 1987; McKay et al 1999), in Sweden 23.4-38.2 (Appelros and Borgstrom 1999; Sandzen et al 2009), in the Netherlands 16 (Eland et al 2000), in Germany 20 (Lankisch et al 2002), in the USA 43.8 (Frey et al 2006), and in Finland it is one of the highest 73.4-102 (Jaakkola and Nordback 1993; Pelli et al 2009) per 100,000 inhabitants. The cause of this increased incidence has been speculated: for instance, increased alcohol consumption, greater accuracy of diagnosis, and an increased prevalence gallstones and obesity have been suggested to be possible causes (Jaakkola and Nordback 1993; Appelros and Borgstrom 1999; Lindkvist et al 2004). The mean age at the first attack of AP is 60 years, and the sex distribution is almost equal (Corfield et al 1985; Thomson et al 1987; Yadav and Lowenfels 2006). The mortality from AP has been reported to have decreased (Jaakkola and Nordback 1993; Lankisch et al 1996; McKay et al 1999) or to be stable. (Gronroos and Nylamo 1999) Increasing age is associated with higher mortality (Corfield et al 1985; McKay et al 1999). However, the higher mortality in elderly persons has been found to be associated with concomitant medical or surgical diseases, rather than complications of AP (Fan et al 1988). The mortality rate of patients with recurrent disease has been found to be lower than that of patients with first attacks (Appelros and Borgstrom 1999), and the proportion of recurrent attacks of all cases has been as high as 21% (Cavallini et al 2004).

2.3 Etiology of acute pancreatitis

The most common etiology of AP is alcohol abuse or gallstones, comprising about 70-80% of all cases (Forsmark et al 2007). In up to 10% of the cases, the cause of AP still remains unknown (idiopathic AP) (Tonsi et al 2009), and other causes (e.g. hypercalcemia, hypertriglyceridemia, trauma, drugs, infections, endoscopic retrograde cholangiopancreatography (ERCP), developmental abnormalities, tumors, hereditary and autoimmune causes) are uncommon or controversial (Kemppainen and Puolakkainen 2007; Tonsi et al 2009; Wang et al 2009). The predominant etiology varies in different countries (Corfield et al 1985; Lankisch et al 1996; Appelros and Borgstrom 1999; Cavallini et al 2004; Lindkvist et al 2004; Frey et al 2006; Yadav and Lowenfels 2006). Alcohol is the most common cause of AP in Finland, and alcohol consumption correlates with the incidence of AP (Jaakkola and Nordback 1993). In Sweden, gallstones are the most common cause in first attacks (Lindkvist et al 2004) and alcohol in recurrent attacks of AP (Appelros and Borgstrom 1999; Sandzen et al 2009). AP caused by alcohol has been found to be more common in men, gallstone AP in women, and idiopathic AP is similar in both sexes (Yadav and Lowenfels 2006; Tonsi et al 2009).

2.4 Diagnosis of acute pancreatitis

The clinical diagnosis of AP is based on characteristic epigastric pain and nausea or vomiting, combined with elevated serum levels of amylase (>3 times the upper reference limit) and/or typical AP imaging findings on CT (Banks et al 2006).

There are no specific and exact laboratory tests for AP, but testing for elevated serum/plasma levels of amylase and/or lipase is used. The measurement of amylase is used more widely, and a level of at least 3 times the upper reference limit is suggested to be the most accurate cutoff (Forsmark et al 2007). In clinical studies, the sensitivity of serum amylase estimation has been 45-85% and specificity 91-99% (Gumaste et al 1993; Kemppainen et al 1997; Treacy et al 2001). Correspondingly, the sensitivity of serum lipase estimation has been 67-100% and specificity 96-97% (Gumaste et al 1993; Treacy et al 2001). Measurements of other enzymes (including pancreatic isoamylase, phospholipase 2, elastase 1 and trypsinogen-2) in serum or urine have also been proposed as diagnostic tools for AP (Forsmark et al 2007). A rapid urinary trypsinogen-2 test strip has been developed; it has proved to be highly sensitive in distinguishing AP patients. A negative result can rule out AP with a very high probability (Kemppainen et al 1997; Kylanpaa-Back et al 2002b).

CT is often used as the gold standard for confirming the diagnosis of AP. In addition, CT is effective in excluding alternative diagnoses, in determining the severity of AP, and in identifying complications (Forsmark et al 2007). The primary role of ultrasonography (US) in the diagnosis of AP is to identify gallstones or dilation of the common bile duct due to choledocholithiasis. (Banks et al 2006) Magnetic resonance imaging (MRI) is as accurate as CT in imaging the pancreas and assessing the stage of severity of AP (Clancy et al 2005; Forsmark et al 2007). (Morgan 2008)

2.5 Severity assessment of acute pancreatitis

On admission to hospital it is difficult to predict whether a given patient's disease will take a mild or a severe course. This would, however, be most important so as to screen patients with severe disease and treat them in the intensive care unit. A variety of predictive systems have been developed for this purpose, including the measurement of markers in serum and urine (Mayer et al 2000; Granger and Remick 2005; Beger and Rau 2007), CT (Balthazar et al 1990), and multiple factor scoring systems. Most of the scoring systems are unfortunately too complicated, insufficiently sensitive, or not available soon enough.

There are some clinical predictors and laboratory markers of poor outcome in AP. High age is one predictive factor for mortality (Corfield et al 1985; McKay et al 1999), and early and persistent (>48 h) OF is another predictive factor for mortality (Isenmann et al 2001; Johnson and Abu-Hilal 2004; Beger and Rau 2007). Obesity is a risk factor for severe AP (Martinez et al 2006), and pancreatic necrosis is a risk factor for severe outcome as well (Balthazar et al 1990; Simchuk et al 2000). The role of early and/or sustained hemoconcentration in predicting severe AP and/or OF has also been studied in humans. Baillargeon et al. (Baillargeon et al 1998) found that hemoconcentration with an admission hematocrit >/= 47% or failure of admission hematocrit to decrease at 24 h were risk factors for the development of pancreatic necrosis. However, these hematocrit values were not predictive of OF. There are other studies with similar results (Brown et al 2000; Gan and Romagnuolo 2004), but also studies with controversial results (Lankisch et al 2001; Remes-Troche et al 2005; Gardner et al 2006). Two of the ones with controversial results (Lankisch et al 2001; Gardner et al 2006) found that the absence of admission hemoconcentration had a strong negative predictive value for necrosis. In one study, a high serum creatinine level (>2.0 mg/dl) and/or marked hyperglycemia (>250 mg/dl) on admission were shown to predict mortality (Blum et al 2001). C-reactive protein (CRP) has been widely used as a predictor of severe AP (cutoff 150 mg/l), but only measurement at 48 h after admission has been shown to be reasonably

accurate (Rettally et al 2003; Mofidi et al 2006). Among the biochemical markers of significance, IL-6, IL-10, procalcitonin (PCT) and trypsinogen-activation peptide (TAP) are most likely to be used in clinical practice as predictors of severity (Kylanpaa-Back et al 2002a; Mofidi et al 2009). A combination of plasma IL-10 and serum calcium measurements has been shown to predict OF with high accuracy at hospital admission (Mentula et al 2005).

Multi-factorial scoring systems have been used to assess severity. These are, e.g.: Ranson's score (Ranson et al 1974), Glasgow/Imrie score (Blamey et al 1984) and the APACHE II score (Knaus et al 1985) based on clinical and laboratory findings. For predicting mortality, organ dysfunction scores (MODS (Marshall et al 1995) and SOFA score (Vincent et al 1998)) have been used. An APACHE II score that rises during the first 48 h is strongly suggestive of the development of severe AP, whereas an APACHE II score that falls within the first 48 h predicts mild AP (Banks et al 2006). One scoring system that rapidly identifies patients with a mild disease form has been developed for harmless acute pancreatitis (HAPS). It is based on three parameters (absence of rebound tenderness/guarding, e.g. no signs of peritonitis, normal serum creatinine level, and normal hematocrit level). The HAPS has shown high specificity (97%) and positive predictive value (98%), but quite low sensitivity (29%) and a negative predictive value (20%). (Lankisch et al 2009)

2.6 Pathogenesis of acute pancreatitis and clinical manifestations

Although alcohol abuse and gallstones account for the majority of the cases of AP, the exact mechanisms by which these factors initiate AP are presently unknown. Despite the etiological factors in the pathogenesis of AP, the initial insult is probably the premature intracellular activation of digestive enzymes (proteases) leading to autodigestion of pancreas. The disease progression is a three-phase continuum: local inflammation of the pancreas, a generalized inflammatory response (SIRS), and the final stage of MOF in the most severe form of AP (Makhija and Kingsnorth 2002). (Bhatia et al 2005)

The most common theory is that AP develops as a result of an injury of acinar cells, consequently permitting the leakage of pancreatic enzymes (trypsin, chymotrypsin and elastase) into pancreatic tissue, where they become activated and initiate autodigestion of pancreas. The activated proteases (trypsin, elastase and lipase) break down tissue and cell membranes, causing edema, vascular damage, hemorrhage, necrosis and a local inflammatory reaction. The key propagating factor is thought to be microvascular derangement (including vasoconstriction, shunting, inadequate perfusion, increased

permeability, and increased blood viscosity, coagulation, and adhesion and activation of leukocytes that can cause occlusions in the venules and microthrombus formation), the extent of which influences also the severity of the disease. These processes may be caused or exacerbated by an ischemia-reperfusion injury and the development of oxygen-derived free radicals. (Cuthbertson and Christophi 2006)

Derangement in the coagulation cascade is likely to be at least partially due to systemic inflammation, but it may also represent a coagulation failure that parallels MOF.

The current knowledge of the pathogenesis of AP is based mostly on studies using animal models. Because the clinical disease varies widely in regard to its course and severity, and there is hardly no access to examine the pancreatic tissue during AP, several animal models have been developed. In these models AP is caused by parenteral administration of cholecystokinin analogues or arginine, pancreatic duct obstruction, bile acid perfusion of the pancreatic duct, a choline-deficient and ethionine-supplemented diet, and a combination of an alcohol diet and parenteral administration of cholecystokinin analogues. (Pandol et al 2007)

2.6.1 Zymogen activation

The pancreatic digestive enzymes are synthesized and stored as inactive zymogens in acinar cells. Normally these enzymes are secreted into the duodenum, where the intestinal endopeptidase (enterokinase) hydrolyses trypsinogen, releasing TAP and activating trypsin. In the secretory granules of acinar cells, the autoactivation of trypsinogen is inhibited by pancreatic secretory trypsin inhibitor (PSTI). (Naruse 2003) The intra-acinar activation of zymogens is a key event in the pathogenesis of AP (**Figure 2**). Several pathways are thought to be involved in the intracellular conversion of pancreatic zymogens to active enzymes. These include: 1) trypsinogen autoactivation to trypsin, 2) cleavage of trypsinogen to trypsin by lysosomal hydrolase cathepsin B (CTBS), 3) diminished activity of intracellular pancreatic trypsin inhibitory, 4) leakage of zymogens and lysosomal enzymes into the cytoplasm, and subsequent proteolytic activation, 5) shunting of zymogens into membrane-bound compartments that contain active proteases, 6) uptake and processing of secreted zymogens by endocytic pathways, and 7) enhanced susceptibility of zymogens to proteolysis because of oxidation (Bhatia et al 2005). The first three of these pathways have been studied most extensively. Also the intracellular elevation of calcium is believed to be, at least, a cofactor in the zymogen activation of AP (Weber and Adler 2003). In AP there are two

pathways of the acinar cell death: necrosis and apoptosis (mediated by activation of caspases). The conversion of the cell death response to apoptosis has been associated with improvement in the severity of AP. (Pandol et al 2007)

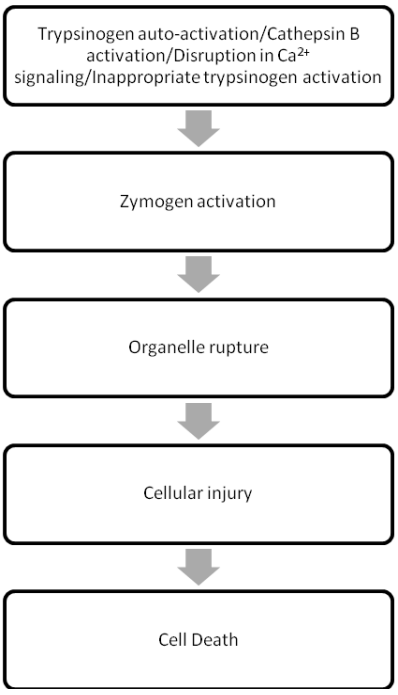

Figure 2. Intra-acinar zymogen activation.

2.6.2 Systemic inflammation

In the pathogenesis of AP, a local injury and inflammation in the pancreas can proceed to systemic inflammation causing SIRS (**Figure 3**). This is characterized by abnormal body temperature, tachycardia, tacypnoe and abnormal leukocyte count (Bone et al 1992). Normally, the host inflammatory response is confined to the injured interstitial space by localization of proinflammatory mediators to the affected area, and by various inhibitors. However, if this response is activated in an uncontrolled fashion and disseminated via the circulation (becoming systemic), organs distant from the initial insult can be affected, leading to multiple organ dysfunction syndrome (MODS). In SIRS many proinflammatory mediators (cytokines), nitric oxide (NO) and components of the complement are released. Polymorphonuclear neutrophils (PMN) release free radicals and proteolytic enzymes, and contribute to the initiation and perpetuation of the inflammatory response. (Norman 1998; Granger and Remick 2005) In addition to this, cellular and complement-mediated cytotoxic

injury, the sequestration of platelets, leukocytes and erythrocytes in the microvascular circulation, cause ischaemic injury. Activation of immunoeffector cells and upregulation of proinflammatory cytokines activates also the vascular endothelium, which, in turn, increases the expression of cell surface adhesion molecules and produces inflammatory mediators. (**Figure 8**, page 46) (Wilson et al 1998) In systemic inflammation, an excessive proinflammatory burst is rapidly followed by an anti-inflammatory reaction that may result in immune suppression (Mentula et al 2003), a compensatory anti-inflammatory response syndrome (CARS) (Makhija and Kingsnorth 2002).

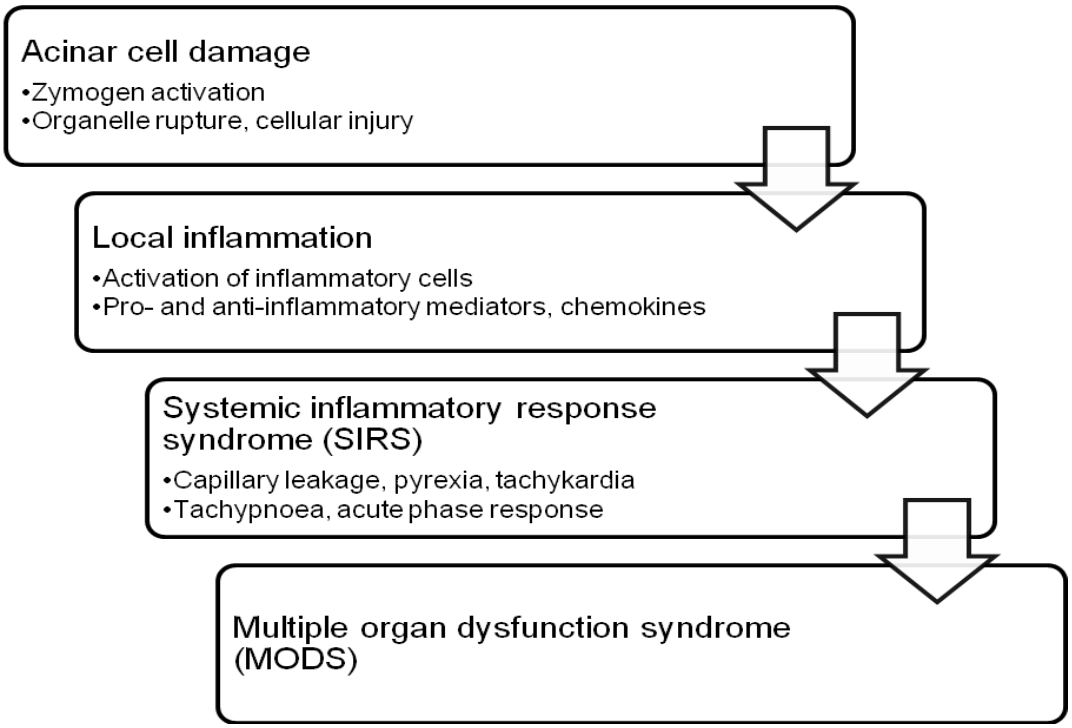

Figure 3. Inflammatory cascade in acute pancreatitis.

2.6.2.1 Inflammatory cells

The infiltration of inflammatory cells into the pancreas is an early and central event in AP, and promotes local injury and aggravates systemic complications of the disease. The acinar, ductal and pancreatic stellate cells play a dynamic role in leukocyte attraction via secretion of chemokines and cytokines and expression of adhesion molecules (Bhatia et al 2005). The leukocyte movement (adhesion to the blood vessel wall, transmigration through the blood vessel wall and infiltration into the parenchyma) is the central event in the pathogenesis of AP. The first step in the leukocyte movement is the rolling of leukocytes on the surface of

endothelial cells. This phenomen is mediated by selectins, which are a family of cell surface molecules on leukocytes and endothelial cells. After rolling, the leukocytes adhere tightly to the endothelial cells. This adhesion is mediated by intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 expressed on endothelial cells, and by CD11b-CD18, CD11a-CD18 and integrin alpha4beta1 expressed on neutrophils and monocytes. (Repo and Harlan 1999) After adhesion, the leukocytes transmigrate through the endothelial barrier into the interstitium, and this is mediated by platelet endothelial cell adhesion molecule (PECAM)-1, CD99, junctional adhesion molecules (JAM) and vascular endothelial (VE)-cadherin. Finally, the leukocyte movement through the tissues is guided by interactions between leukocyte integrins and components of the extracellular matrix (e.g. fibronectin, vitronectin, collagen and laminin). (Radi et al 2001; Vonlaufen et al 2007)

Neutrophils

Neutrophils are attracted into the pancreas and other tissues by chemokines (e.g. interleukin (IL)-8), platelet-activating factor (PAF) and leukotriene B4 (LTB4). Activated neutrophils can release myeloperoxidase, proteases (e.g. elastase) and reactive oxygen species (ROS) into the interstitium and damage it. (Vonlaufen et al 2007)

Monocytes and macrophages

The infiltration of monocytes and macrophages in the pathogenesis of AP is similar to the infiltration of neutrophils. The activated monocytes and macrophages release ILs-1, -6, -8, tumor necrosis factor (TNF)-alpha and ROSs and cause damage. (Vonlaufen et al 2007)

Lymphocytes

In experimental studies lymphocytes have also been found to infiltrate into pancreatic tissue and to release cytokines.

2.6.2.2 Mediators of inflammation

Proinflammatory mediators believed to participate in the pathogenesis of AP include: TNF-alpha, IL-1beta, IL-6, PAF, ICAM-1, IL-8, growth-related oncogene-alpha/cytokine-induced neutrophil chemoattractant (GRO-alpha/CINC), monocyte chemoattractant protein (MCP)-1, and substance P.

Anti-inflammatory mediators in AP include: IL-10, complement component C5a, soluble TNF receptors (sTNFR), IL-1 receptor antagonist (IL-1ra), and neutral endopeptidase (NEP).

The expression of these mediators is regulated by transcription factors (e.g. nucler factor kappa B, NF-KB) (Makhija and Kingsnorth 2002). (Bhatia et al 2000; Makhija and Kingsnorth 2002; Bhatia et al 2005; Granger and Remick 2005)

Tumor necrosis factor-alpha

TNF-alpha is a polypeptide and predominantly macrophage-derived early phase cytokine which interacts via a specific receptor TNFR. TNF is also released by neutrophils and acinar cells (Bhatia et al 2005). It has a short plasma half-life, 14-18 min, due to the rapid clearance of the liver, gastrointestinal tract and kidney. TNFRs are present on many cells and can be released into the circulation as soluble TNFR (sTNFR) by binding of TNF. TNF activates neutrophils, upregulates the endothelial adhesion molecules E-selectin and ICAM-1, enhances the endothelial expression of tissue factor (TF), increases capillary permeability, and has a direct toxic effect on cells. (Conway and Rosenberg 1988; Wilson et al 1998; Makhija and Kingsnorth 2002) In severe AP, high levels of TNF have been found in clinical and experimental studies (Exley et al 1992; Grewal et al 1994)

Interleukin-1

IL-1 is an early phase cytokine which is produced by many different cell types, predominantly by macrophages, but to a lesser extent also by acinar cells (Bhatia et al 2005). It interacts via an IL-1 receptor. It can activate neutrophils and endothelium by upregulation of all classes of adhesion molecules. It causes fever, hypotension, increased capillary permeability, and a release of other cytokines (Makhija and Kingsnorth 2002). (Wilson et al 1998)

Interleukin-6

IL-6 is a phosphoglycoprotein and an early phase cytokine which interacts via an IL-6 receptor. It is produced by monocytes, endothelial cells, smooth muscle cells, fibroblasts and periacinar myofibroblasts (Makhija and Kingsnorth 2002; Bhatia et al 2005). It induces hepatic synthesis of acute phase proteins (e.g. CRP), stimulates T-cell differentiation and promotes proliferation of B-cells. (Wilson et al 1998)

Interleukin-8

IL-8 is a chemokine which is released mainly by macrophages and endothelial cells. It interacts via an IL-8 receptor. It is chemotactic for neutrophils and stimulates their activation. (Wilson et al 1998; Makhija and Kingsnorth 2002)

Interleukin-10

IL-10 is an anti-inflammatory cytokine which upregulates IL-1ra and sTNFR production and reduces IL-8 and MCP-1 levels (Makhija and Kingsnorth 2002; Bhatia et al 2005).

Phospholipase A_2

Phospholipase A_2 (PLA2) is a group of enzymes which occur in a membrane-associated form or in an extracellular (secretory) form. The membrane-associated form generates secondary messengers and catalyses the hydrolysis of phospholipids. (Wilson et al 1998) High extracellular PLA2 levels have been found in AP patients with SIRS (Hietaranta et al 1999).

Platelet-activating factor

PAF is a low molecular weight phospholipid which acts via a specific cell surface receptor. It is released by inflammatory cells, such as endothelial cells, macrophages and neutrophils (Makhija and Kingsnorth 2002). It induces aggregation of platelets and PMN, systemic vasodilatation and increased endothelial permeability. (Wilson et al 1998) PAF is inactivated by the enzyme PAF acetylhydrolase (PAF-AH) (Bhatia et al 2005).

2.6.2.3 Complement and CD59

Complement has a central role in the innate immune system, and is composed of more than 30 proteins in plasma and on cell membranes. It has three main activities: 1) to defend against bacterial infection (opsonization, chemotaxis and activation of leukocytes, and lysis of bacteria and cells), 2) to bridge innate and adaptive immunity, and 3) to dispose of immune complexes and the products of inflammatory injury and apoptotic cells. (Walport 2001a)

2.6.2.3.1 Activation of complement

Complement can be activated by three pathways: 1) the classical, 2) mannose-binding lectin (MBL), and 3) alternative pathways (**Figure 4**). (Walport 2001a; Cole and Morgan 2003) The classical pathway begins when antibody binds to a cell surface, and the C1 proteins (composing of one C1q, and two C1r and C1s units) C1q unit binds to antibody. After that C1r and C1s are activated, and C1s cleaves C4 protein to C4a and C4b, and C2 protein to C2a and C2b forming C4bC2a convertase. C4bC2a then cleaves C3 protein to C3a and C3b, and C5 protein to C5a and C5b. C5b forms the C5b-C9 complex with proteins C6, C7, C8 and several C9 proteins; it is the membrane-attack complex (MAC) (Podack and Tschopp 1984). The lectin-pathway is similar to the classical pathway, and it begins when MBL binds

to mannose groups on the bacterial surface, and its mannose-binding lectin-associated serine proteases (MASP)-1 and MASP2 activate C2 and C4 proteins. After that the pathway is the same as the classical pathway. The alternative pathway is, in minimal extent, activated at all times. C3 protein can be hydrolyzed to C3(H_2O) and can bind to factor B, which can be cleaved to Bb by factor D. This formed C3(H_2O)Bb complex can cleave C3 protein to C3a and C3b. Factor D cleaves factor B to Bb, which forms the C3bBb convertase with C3b. Factor P (properdin) stabilizes C3bBb. This C3bBb convertase is similar to C4bC2a convertase, and can, in turn, cleave C3 and C5 proteins. The forming C5b unites with C6-C9 proteins, composing the MAC.

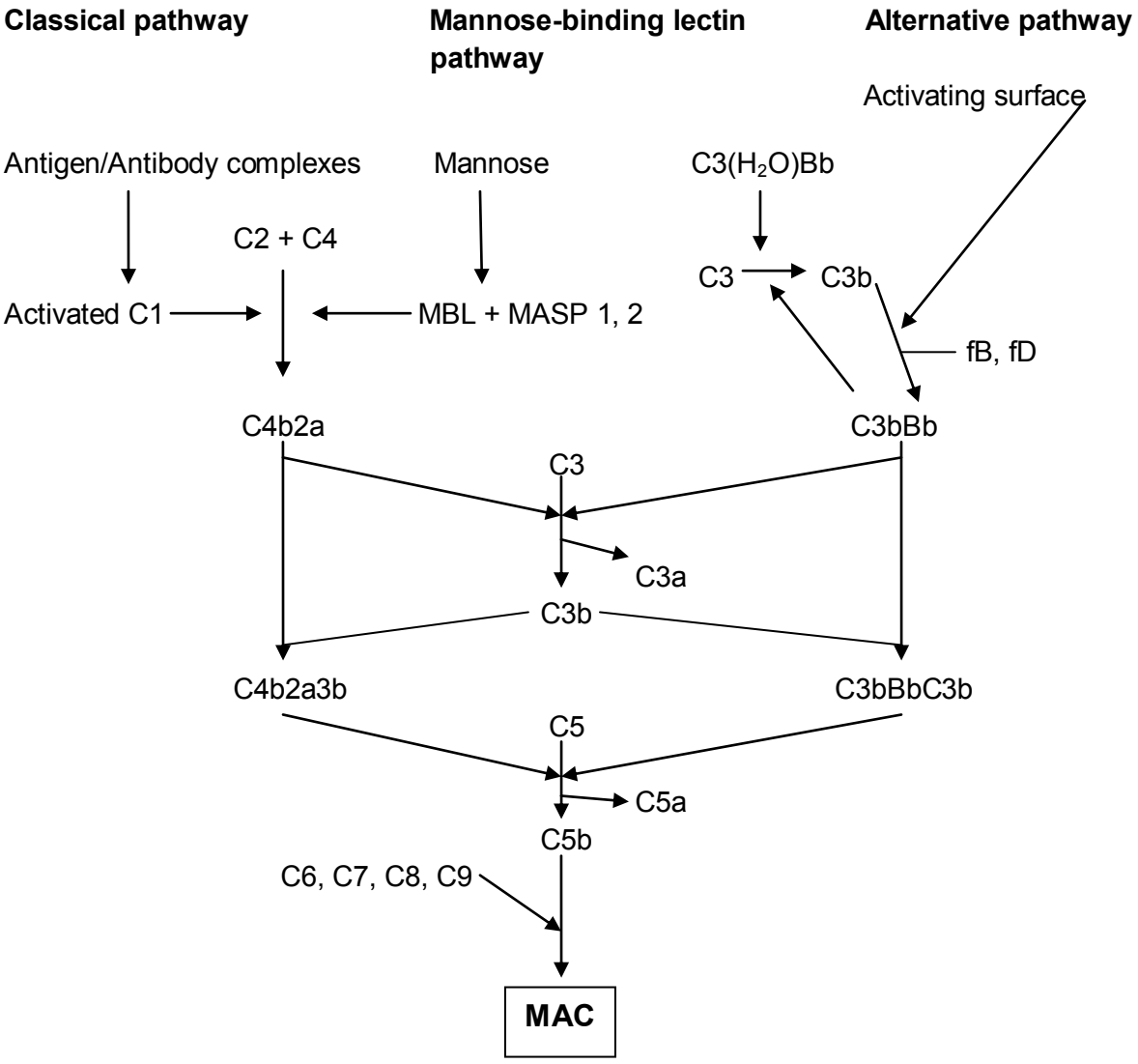

Figure 4. Complement activation pathways, (MBL=mannose-binding lectin, MASP=mannose-binding lectin-associated serine protease, fB=factor B, fD=factor D, MAC=membrane-attack complex).

The main function of MAC is to destroy invading organisms and, possibly, malignant cells by comprising a transmembrane channel and forming ultrastructural membrane lesions (Podack and Tschopp 1984; Morgan 1999). In the activation of complement, by cleavage of C3 and C5, small fragments, i.e. C3a and C5a, are released. They have powerful anaphylactic properties (on phagocytic cells, mast cells and basophils) and C5a has also chemotactic properties (on leukocytes) (Morgan 1999; Cole and Morgan 2003). In the activation of complement, also C3b and C4b are released, and they can opsonize pathogens for phagocytosis (Cole and Morgan 2003).

When tissue injury occurs, complement can be activated through immune complexes, and ischemia and reperfusion, which expose mitochondrial proteins and phospholipids (neoantigens) (Barrington et al 2001). Necrotic cells also lack the regulatory proteins, which normally inhibit complement binding. The activated complement can cause damage through MAC and by activating leukocytes bearing complement receptors for C3b and C4b. It can also amplify the tissue injury by activating inflammatory cells via anaphylatoxins C5a and C3a. (Walport 2001b; Cole and Morgan 2003) MAC has also proinflammatory properties on nucleated host cells and endothelial cells by inducing the release of inflammatory mediators, secretion of cytokines, degranulation and proliferation of these cells (Morgan 1999; Cole and Morgan 2003). In an *in vitro* study it was shown that coagulation factors Xa and XIa, plasmin and thrombin can cleave C3 and C5 to generate C3a and C5a (Amara et al 2008). Products of complement activation have been found to collaborate with the adaptive immune system to enhance responses to antigens (Cole and Morgan 2003). Complement has also anti-inflammatory properties; it clears immune complexes from tissues, and binds to apoptotic cells helping to eliminate them (Walport 2001b).

2.6.2.3.2 Regulation of complement

Complement is regulated by many mechanisms which are balanced so that the activation of complement takes place on the surface of invading microorganisms, and the deposition of complement on normal cells is limited. Deficiencies of complement or its regulator proteins, as well as disturbances in the complement regulator mechanisms can cause many diseases, e.g. susceptibility to pyogenic infections, hemolytic-uremic syndrome, glomerulonephritis, and systemic lupus erythematosus. (Walport 2001a)

Several proteins in the plasma and on cell membranes downregulate the activation of complement (**Table 1**). In the plasma, C1 inhibitor (C1inh) inhibits the function of C1, and C4b binding protein (C4bp), and factors H and I inhibit C3C5 convertase. On cell

membranes, the decay accelerating factor (DAF), membrane cofactor protein (MCP), and complement receptor (CR)-1 inhibit also C3C5 convertase, and CD59 inhibits C5b-C9 complex (Barrington et al 2001). (Morgan 1999) The function of MAC-controlling proteins is to restrict lysis to target membranes and to prevent lysis of innocent bystander or host cells. These proteins include also C5b-7 inhibitors and S-protein, which inhibits C9 polymerization and channel formation. (Podack and Tschopp 1984) Nucleated cells (e.g. neutrophils) can also remove MACs from the cell membrane by Ca-dependent shedding or endocytosis. C5a and C3a are inactivated by a plasma enzyme called carboxypeptidase N (CPN). (Morgan 1999)

Table 1. Complement regulatory proteins, (C1inh=C1 inhibitor, CPN=carboxypeptidase N, MCP=membrane cofactor protein, DAF=decay-accelerating factor, CR1=complement receptor 1).

Molecule	**Target or function**
Plasma	
C1inh	C1 and C4Bb
factor H	C3/C5 convertase
factor I	C3/C5 convertase
C5b-C7 inhibitors	inhibit channel formation of MAC
S-protein	inhibit channel formation of MAC
CPN	C3a and C5a
Membrane	
MCP	C3/C5 convertase
DAF	C3/C5 convertase
CR1	C3/C5 convertase
CD59	C5b-C9 complex

2.6.2.3.3 Complement and acute pancreatitis

Pancreatic proteases (trypsin) can activate complement; this has been shown in AP (Foulis et al 1982; Whicher et al 1982; Acioli et al 1997; Hartwig et al 2001). In sepsis, trauma and AP complement activation has been found to be associated with the development of pulmonary damage, ARDS (Jacob and Hammerschmidt 1982; Duchateau et al 1984).

High plasma levels of C3a and high peritoneal fluid levels of C3a, C5a and C5b-C9 complex have been found in AP patients (Bengtsson et al 1990), and high plasma C3a, C5a and C5b-C9 levels in patients with severe AP (Roxvall et al 1990). When AP patients were treated intraperitoneally with protease inhibitor (aprotinin) the C3a levels were lower and the C1INH

levels higher than in control patients (Berling and Ohlsson 1996). In a human study C3 catabolism was increased in AP patients and a falling level of C3 was associated with a fatal outcome (Foulis et al 1982). In one clinical study, high levels of C3a and C5b-C9 were observed to predict severe AP (Gloor et al 2003). In a clinical study, high levels of complement inhibitor protein (C1INH and factor H) were found, but there was no significant association with severity of AP (Whicher et al 1982). Children with severe AP and complement activation after allogeneic hematopoietic stem cell transplantation were treated with C1 esterase inhibitor, which contributed to rapid clinical stabilization (Schneider et al 1999). An experimental AP study revealed that trypsin-generated complement activation takes part in the upregulation of adhesion molecule Mac-1 and in the down-regulation of L-selectin on neutrophils (Hartwig et al 2001). In C5-deficient mice, edema formation in AP was decreased compared to C5-sufficient mice (Merriam et al 1997).

2.6.2.3.4 CD59

CD59 is a 20 kDa cell membrane protein which is expressed on all circulating cells, endothelia, epithelia, and in most organs (Meri et al 1996). It is linked to the membrane by a glycosyl phosphatidylinositol (GPI) anchor and can be removed from cells by phosphatidylinositol-specific phospholipase C (PIPLC). Urine, seminal plasma, cerebrospinal fluid, amniotic fluid and breast milk also contain fluid-phase forms (lacking the GPI anchor) of CD59, i.e. the soluble CD59. These isoforms display specific binding activity towards C5b-C9. However, because of the absent phospholipid tail, they have limited ability to inhibit MAC on cell membranes. (Morgan 1999) CD59 can be shed from epithelial cells either in the form of small membrane vesicles, or after cleavage by phospholipase C or D. (Meri et al 1996)

CD59 binds to C8 and C9, and inhibits the forming of MAC and the transmembrane channel. It has been shown to act as an adhesion molecule for T cells, and it has also been proposed to have a role in cell activation (Wang et al 2002). (Morgan 1999)

In humans CD59 deficiency, due to a somatic gene mutation, is known. It causes a disease called paroxysmal nocturnal hemoglobinuria (PNH). In this disease erythrocytes and platelets lack CD59 and are highly sensitive to autologous complement-mediated lysis and activation, resulting in hemolytic anemia and thrombosis. (Baalasubramanian et al 2004) High plasma CD59 levels have been found in patients with acute myocardial infarction (Vakeva et al 2000). CRP-induced upregulation of complement inhibitor proteins (DAF, MCP and CD59) was found on human endothelial cells, and these proteins were functionally effective in reducing complement-mediated cell lysis (Li et al 2004). In an animal rheumathoid arthritis

model, recombinant soluble CD59 was found to suppress the disease markedly (Fraser et al 2003).

2.6.2.4 High mobility group box 1 protein, a receptor for advanced glycation end products and a soluble form of receptor for advanced glycation end products

High mobility group box 1 protein

High mobility group box 1 (HMGB1) is a 30 kDa nuclear and cytosolic protein which is produced by nearly all cell types (Limana et al 2005). It is composed of three domains: two homologous DNA-binding motifs, A box and B box, and a negatively charged C terminus (Yang et al 2005). In inflammation, it is produced by activated macrophages, and can be released actively (after endotoxin or endogenous cytokine stimulation) by these cells, or passively by necrotic cells. (Wang et al 2004a)

HMGB1 can bind to the receptor for advanced glycation end products (RAGE) (Rouhiainen et al 2004) and Toll-like receptors (TLRs) 2 and 4 (Li et al 2006; Ito et al 2007).

HMGB1 is a cytokine mediator of inflammation and, in particularly, a late mediator of sepsis (it is secreted 20 h post-stimulation) (Wang et al 2004a; Mantell et al 2006). It acts also as a transcription cofactor and a growth factor (Bustin 1999; Limana et al 2005). It can activate macrophages, monocytes and neutrophils to release proinflammatory cytokines, and endothelial cells to upregulate adhesion molecules. On epithelial cells it stimulates barrier failure, and on adherent cells migration (Mitola et al 2006). HMGB1 also mediates transendothelial migration of monocytes (Rouhiainen et al 2004).

Increased serum levels of HMGB1 have been found in patients with disseminated intravascular coagulation (DIC) (Hatada et al 2005), hemorrhage (Kim et al 2005), sepsis (Eriksson 2005; Sunden-Cullberg et al 2006; Ito et al 2007) and acute lung injury (Ueno et al 2004). In experimental sepsis studies, high levels of HMGB1 have been found; the administration of recombinant HMGB1 has induced symptoms of sepsis, and inhibition of HMGB1 has prevented endotoxin- and bacteremia-induced MOF (Yang et al 2005; Mantell et al 2006). In an animal model of thrombin-induced DIC, HMGB1 induced TF expression on monocytes and inhibited protein C (PC) activation, mediated by the thrombin-thrombomodulin (TM) complex (Ito et al 2007). TM has been shown to bind to HMGB1 and

prevent its proinflammatory effects (Abeyama et al 2005). In an animal study, HMGB1 pretreatment was found to decrease liver damage after ischemia/reperfusion (Izuishi et al 2006). The plasma levels of HMGB1 in patients with DIC and organ failure were significantly higher than in patients with DIC but without organ failure, and also higher in non-survivors than in survivors (Hatada et al 2005). In one clinical study, elevated serum HMGB1 levels were found in AP patients (Yasuda et al 2006).

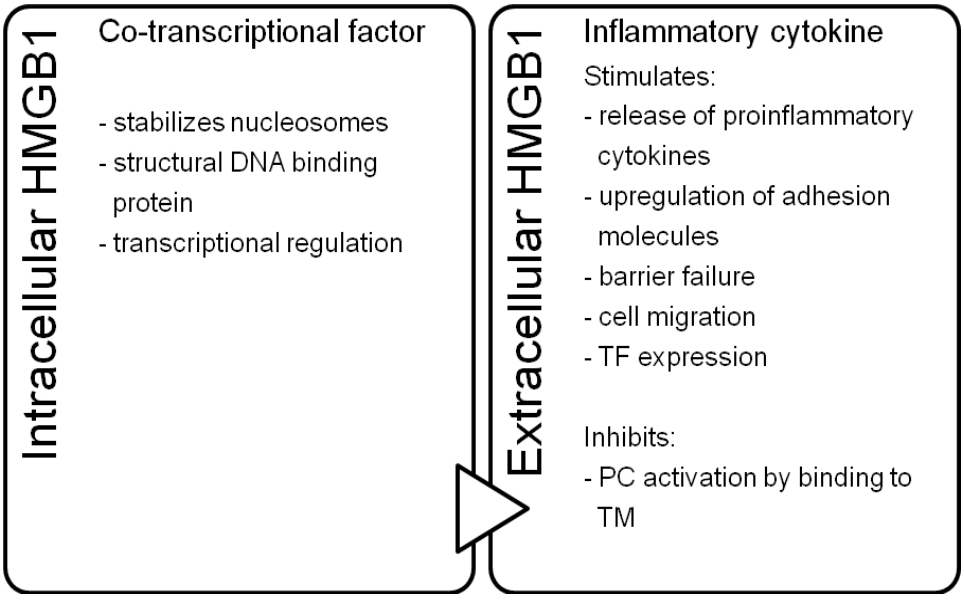

Figure 5. Intra- and extracellular functions of HMGB1, (TF=tissue factor, PC=protein C, TM=thrombomodulin).

Receptor for advanced glycation end products and soluble form of receptor for advanced glycation end products

RAGE is a member of the immunoglobulin superfamily, and is expressed on endothelium, neurons, vascular smooth muscle cells and on mononuclear phagocytes (Abeyama et al 2005; Li et al 2006).

RAGE have many ligands, e.g. advanced glycation end products (AGEs), HMGB1, members of the S100 family and amyloid peptide. The binding of a ligand activates the NF-KB signaling and the mitogen-activated protein kinase (MAPK) pathways (Wang et al 2004a). This ligand binding induces cell migration, cell invasion, tumor growth and metastasis (Mitola et al 2006).

A soluble form of RAGE (sRAGE) is present in the circulation (Yonekura et al 2003); it comprises the extracellular domain of RAGE (Bierhaus et al 2005), and preserves its original ligand-binding capacity (Schmidt et al 2000). The circulating pool of sRAGE consists of a splice variant, named endogenous secretory RAGE (esRAGE) and proteolytically cleaved forms of RAGE (Hudson et al 2005). Elevated levels of sRAGE have been found in disorders such as end-stage renal disease (Kalousova et al 2007) and acute lung injury (Uchida et al 2006), and reduced levels have been found in rheumathoid arthritis (Pullerits et al 2005), Alzheimer's disease (Emanuele et al 2005) and essential hypertension (Geroldi et al 2005).

2.6.3 Coagulation and hemostatic factors

Hemostasis involves vessel wall and endothelial cells, soluble plasma proteins (coagulation proteins and their regulators), cellular components within the vessel lumen (red blood cells, platelets and leukocytes), and microparticles derived from platelets and leukocytes (Furie and Furie 2007). It is a physiologic process which controls the blood fluidity and, at the same time, has the capacity to produce a hemostatic plug outside a damaged blood vessel. Thrombosis refers to the same event inside the vessel lumen, and consists of platelet accumulation, adhesion, activation and aggregation, as well as tissue-factor-initiated thrombin generation and fibrin formation (Furie and Furie 2007). Blood coagulation, a precise and balanced thrombin generation at sites of vascular damage, is induced by adherent platelets (Butenas and Mann 2002). In healthy persons, the hemostatic process is counter-balanced by a system of anticoagulant mechanisms which ensure that the hemostatic effect is regulated, and does not extend inappropriately. Normally these anticoagulant factors are slightly dominating. (Dahlback 2000) In pathologic states (e.g. systemic inflammation), however, these hemostatic events can escape the control mechanisms, and this leads to thrombosis.

2.6.3.1 Models of coagulation

Previously, the 'Waterfall' or 'Cascade' model of coagulation divided it into extrinsic and intrinsic pathways, which united at the level of factor X, forming a common pathway (**Figure 6**) (Davie and Ratnoff 1964; Macfarlane 1964). According to this model, blood coagulation involves a wide series of coordinated and calcium-dependent conversions of proenzymes to the respective serine proteases, culminating finally in the conversion of prothrombin into thrombin (Dahlback 2000). In the extrinsic pathway after vascular damage, TF is exposed to

the circulation and binds to and activates factor VII to FVIIa. The TF-FVIIa complex activates both FIX and FX to FIXa and FXa. In the intrinsic pathway on negatively charged surfaces, contact activation of factor XII occurs in the presence of prekallikrein and high molecular weight kininogen. The FXIIa then activates factor XI to XIa, which in turn activates factor IX to FIXa. The FIXa further activates factor X with factor VIIIa. In the common pathway, FXa forms a prothrombinase complex with factor Va, which then leads to the conversion of prothrombin to thrombin. Thrombin further converts fibrinogen to fibrin. Within this cascade model, the contribution of primary hemostasis by platelets was considered to be an independent mechanism (Adams and Bird 2009). Prothrombin time (PT) measures the extrinsic pathway, and activated partial thromboplastin time (aPTT) measures the intrinsic pathway (Tanaka et al 2009).

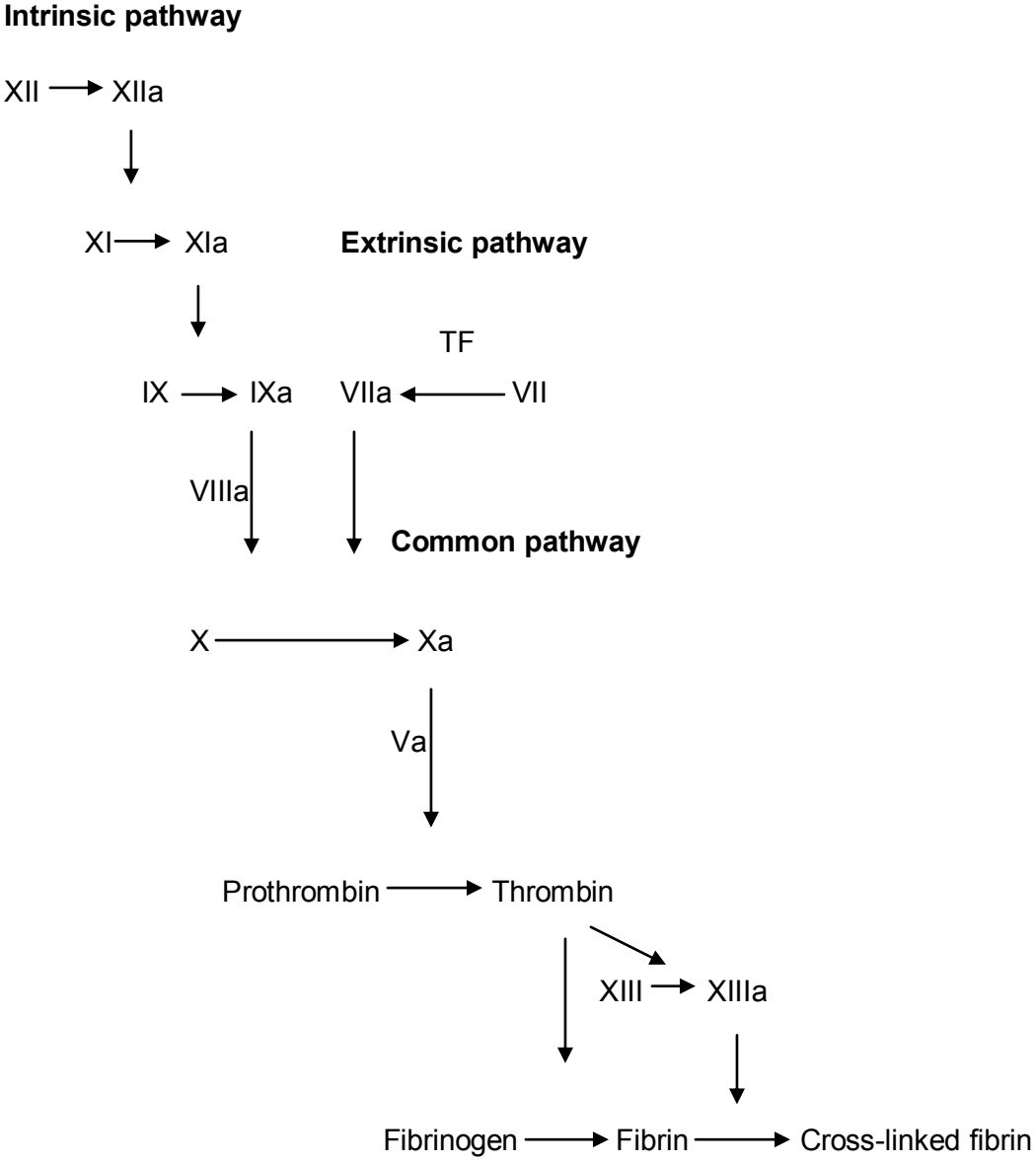

Figure 6. The cascade model of coagulation, (TF=tissue factor).

The cascade model was replaced, almost a decade later, by a cell-based model of coagulation (**Figure 7**) (Hoffman and Monroe 2001). In this model, coagulation is believed to occur in three overlapping stages: 1) initiation, 2) amplification, and 3) propagation (Hoffman and Monroe 2001). In the initiation phase, TF is exposed to coagulation factors either by TF-bearing cells or by damage to endothelium. TF forms a complex with factor VIIa on the phospholipid surface of the cell membrane, after which, this complex activates factors IX and X. The activated factor X forms a complex with Va, and this complex further converts prothrombin to thrombin. In the amplification phase, platelets and cofactors are activated to generate more thrombin, which, in turn, activates more platelets and cofactors enhancing the

thrombin-generating potential. Both TF-VIIa and FIXa-FVIIIa complexes activate FX, leading to thrombin generation. In the propagation phase, large amounts of thrombin are generated on the surface of the platelets. This causes a thrombin burst, which further generates fibrin from fibrinogen. The forming thrombin also activates factor XIII and thrombin-activatable fibrinolysis inhibitor (TAFI). The factor XIIIa cross-links with fibrin strands to form a stable fibrin network, and the TAFI protects the forming clot from plasmin-mediated fibrinolysis. (Dahlback 2000; Butenas and Mann 2002)

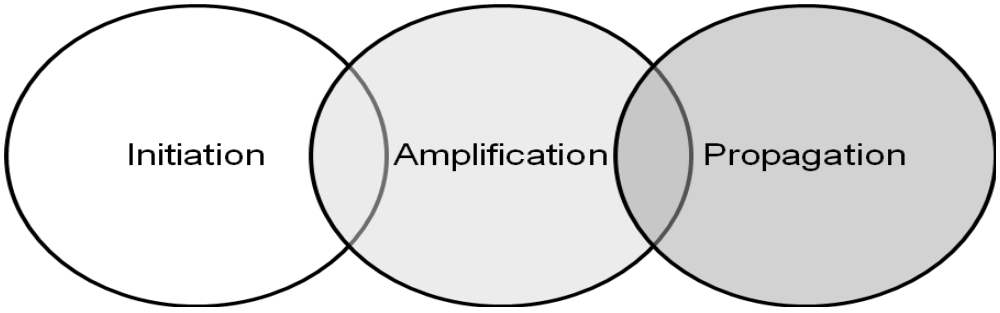

Figure 7. The cell-based model of coagulation.

There is a basic difference between the cascade model and the cell-based model. In the cascade model, the coagulation factors are thought to control and direct coagulation on the cell surfaces, whereas in the cell-based model the cells are thought to regulate the coagulation process by different cell-surface receptors for coagulant factors (Hoffman and Monroe 2001). Furthermore, in the cell-based model also the activated platelets seem to have a more important role, and the coagulation is believed to occur by the overlapping of different phases, rather than in a series of events, as implied in the cascade model.

2.6.3.2 Regulation of coagulation

Regulation of coagulation occurs at each level of the pathway, either by enzyme inhibition or by modulation of the activity of the cofactors (Vine 2009). The natural anticoagulant systems consist of tissue factor pathway inhibitor (TFPI), antithrombin (AT), protein C (PC) pathway, and protein S (PS).

TF is regulated by TFPI (Morrissey 2001), which binds to and inhibits the FXa and the TF-FVIIa complex.

AT binds to and inhibits primarily thrombin, but also factors Xa and IXa (Esmon 2004a; Lippi et al 2009).

PC is activated by thrombin bound to TM, becoming activated protein C (APC), which proteolytically cleaves and inhibits factors Va and VIIIa. PS works as a cofactor for APC. (Esmon 2000; Butenas and Mann 2002)

2.6.3.3 Disseminated intravascular coagulation

DIC is an acquired syndrome characterized by systemic activation of coagulation, which leads to intravascular deposition of fibrin and microvascular thrombosis (Muller-Berghaus et al 1999). Vascular thrombosis, in turn, can contribute to OF. At the same time, the accelerated consumption of platelets and coagulation factors can induce bleeding. The common clinical conditions that are associated with DIC are sepsis, trauma, malignancy, obstetrical complications, severe toxic or immunological reactions, severe hepatic failure, vascular abnormalities, and organ destruction e.g. severe AP. (Taylor et al 2001) The definition of DIC includes overt and non-overt DIC; the former means a stressed, but decompensated hemostatic system, and the latter a stressed but compensated hemostatic system (Taylor et al 2001). The frequency of the occurrence of DIC in different diseases varies considerably, in sepsis overt DIC may occur in 30-50 % and in trauma in 50-70 % of cases. The development of DIC increases the risk of death in the underlying conditions. (Levi and Ten Cate 1999)

The central features in the pathogenesis of DIC include increased formation of fibrin, suppression of the natural anticoagulant systems, and impairment of fibrinolysis. The systemic formation of fibrin is caused by TF-induced and increased thrombin generation. All the natural anticoagulant mechanisms (AT, PC and TFPI) are suppressed in the course of DIC due to the ongoing coagulation, increased levels of proinflammatory mediators (Conway and Rosenberg 1988; Taylor et al 1991), impaired synthesis or their insufficient regulatory capacity (Creasey et al 1993; Mesters et al 1996; Kessler et al 1997). The inhibition of the fibrinolytic system is caused by increased plasma levels of plasminogen-activator inhibitor (PAI)-1. (Levi and Ten Cate 1999)

The diagnosis of DIC is based on a combination of test results and a clinical DIC-associated condition. The scientific subcommittee on DIC of the International Society on Thrombosis and Haemostasis (ISTH) has created a diagnostic scoring system for overt DIC; it is a 5-step algorithm giving a DIC score (**Table 2**). A score of 5 or more is compatible with overt DIC, whereas a score of less than 5 may be indicative (but is not affirmative) for non-overt DIC. (Taylor et al 2001)

In the management of DIC, treatment of the underlying disorder is of crucial importance. However, other supportive measures may be necessary (e.g. transfusion of red blood cells and plasma, or administration of heparin) in the case of severe bleeding or thrombosis.

Table 2. Diagnostic algorithm for the diagnosis of overt DIC according to the Scientific Subcommittee on DIC of the International Society on Thrombosis and Haemostasis.

1) Risk assessment: Does the patient have an underlying disorder known to be associated with overt DIC (e.g. sepsis / severe infection, trauma, organ destruction, malignancy, obstetrical calamities, vascular abnormalities, severe hepatic failure, severe toxic or immunologic reactions)?
   If yes: proceed ; If no: do not use this algorithm

2) Order global coagulation tests (platelet count, prothrombin time, fibrinogen, soluble fibrin monomers or fibrin degradation products)

3) Score global coagulation test results
   - platelet count *(>100=0; <100=1; <50=2)*
   - elevated fibrin-related marker *(no increase=0; moderate increase=2; strong increase=3)*
   - prolonged prothrombin time *(<3 sec.=0; >3 sec. but <6 sec.=1; >6 sec.=2)*
   - fibrinogen level *(>1.0 g/l=0; <1.0 g/l=1)*

4) Calculate score

5) If >/= 5: compatible with overt DIC; repeat scoring daily
   If < 5: suggestive (not affirmative) for non-overt DIC; repeat next 1-2 days

2.6.3.4 Coagulation and inflammation

In critically ill patients, both the inflammatory system and coagulation are activated. There is extensive cross-talk between these two systems, whereby inflammation leads to activation of

coagulation, and coagulation affects inflammatory activity. The key factor in linking these two systems is TF, which is expressed by monocytes after cytokine (IL-6) stimulation, and exposed to blood after endothelial damage (Esmon 1999; Levi and van der Poll 2008). The activation of coagulation by TF leads to thrombin generation and fibrin formation. Thrombin and proinflammatory cytokines (PAF) activate platelets (Dahlback 2000), and this further enhances TF expression (Mackman 2004). Inflammatory mediators also increase platelet production, and the newly formed platelets appear to be more thrombogenic (Esmon 2005b). Simultaneously, the function of natural anticoagulant systems (in particularly PC and AT) is impaired by proinflammatory mediators, and the ongoing thrombin generation leads to their consumption, and their levels thus decrease (Esmon 2000; Levi and van der Poll 2008). In experimental studies, TNF-alpha, IL-1 and endotoxin are found to down-regulate TM and endothelial protein C receptor (EPCR) expression (Esmon 1999). The classical complement pathway regulator protein C4bp binds PS and diminishes its cofactor effect on PC (Vine 2009). Complement activation generates the C5b9 complex, which can cause exposure of phosphatidylserine on the surface of the cells, where it can take part in the initiation and amplification of the coagulation cascade (Esmon 2004a).

Coagulation can also greatly modulate inflammatory activity. The binding of coagulation proteases, e.g. thrombin, TF, and factors VIIa and Xa, can activate protease-activated receptors (PAR 1-4). These receptors have a critical role in inflammation and coagulation. (Coughlin 2000) For example, activation of PARs triggers platelet aggregation and causes endothelium to express numerous adhesion molecules on its surface (Vine 2009). The natural anticoagulant systems also possess many anti-inflammatory properties (Feistritzer and Wiedermann 2007; Levi and van der Poll 2008). EPCR can be shed from endothelium by thrombin and inflammatory mediators, and bind to activated neutrophils, inhibiting their extravasation (Esmon 2004a). Thrombin activates also TAFI, which is an inhibitor of the complement anaphylatoxin C5a, and bradykinin (Esmon 2005a). TM can bind and neutralize HMGB1, which is a late stage mediator of systemic inflammation (Abeyama et al 2005; Esmon 2005b).

2.6.3.5 Coagulation disturbances and acute pancreatitis

Coagulative disorders (mainly consumptive coagulopathy and increased fibrinolysis (Lasson and Ohlsson 1986a; Lasson and Ohlsson 1986b)) are known to occur in severe AP, and they are related to its severity and to OF. In the pathogenesis of pancreatic necrosis, the pancreatic perfusion and hypoxia seem to play an important role. There is accumulating

evidence that microvascular disturbances (vasoconstriction, shunting, increased permeability, inadequate perfusion, and increased blood viscosity and coagulation) are significant events in the progression of AP. Reduced tissue blood flow in AP is related to its severity. (Cuthbertson and Christophi 2006) In severe AP, large vessel hemodynamic changes are common, and also markers for hypovolemia (hemoconcentration, tachycardia, oliguria, azotemia and hypotension) are often observed in the patients on admission to hospital (Pandol et al 2007).

The levels of DIC parameters (low levels of platelets and AT, and high levels of D-dimer) and thrombin-antithrombin complex at admission have been found to be associated with severity and prognosis of AP (Maeda et al 2006). D-dimer levels about four-fold the upper reference limit have been shown to be a sign of severe AP (Salomone et al 2003). The study group of Maeda also found that low PC levels are related to the severity and prognosis of AP (Radenkovic et al 2006). In severe AP, nonsurvivors have been found to have lower PC and AT levels, and higher D-dimer and plasminogen activator inhibitor (PAI)-1 levels than survivors (Radenkovic et al 2004). In one clinical study, the use of recombinant FVIIa for bleeding in pancreatitis showed an improvement in the coagulation parameters, and there were no thromboembolic events (Laffan et al 2005). In Japanese experimental and clinical studies, the continuous regional arterial infusion of antiproteases has been shown to diminish pancreatic necrosis and reduce mortality in severe AP (Takeda 2007). In an animal study, pretreatment with anti-FVIIa reduced IL-6 and macrophage inflammatory protein-2 levels in AP (Andersson et al 2007).

2.6.3.6 Protein C and activated protein C

PC is a vitamin-K-dependent serine protease zymogen, which circulates in plasma (Griffin et al 2006). It is activated by thrombin bound to TM on the endothelial surface, becoming APC, which proteolytically cleaves and inhibits factors Va and VIIIa, and further dampens thrombin generation (Esmon 2000). APC can stimulate fibrinolysis by forming a complex with PAI-1 that leads to inactivation of this fibrinolysis inhibitor (Esmon 2004a). APC can dampen NF-KB signaling in monocytes, decrease the ability of inflammatory mediators to induce TF formation in leukocytes (Esmon 2004b), inhibit neutrophil adhesion to endothelium, and decrease endothelial cell apoptosis (Esmon 2005a; Mosnier et al 2007). PC and APC can bind to EPCR, which enhances their activity. This APC-EPCR complex does not appear to have anticoagulant activity (Esmon 2004a). PS works as a cofactor for APC in inhibiting FVa and FVIIIa. EPCR can be shed from endothelium by thrombin and inflammatory mediators,

and can bind to activated neutrophils inhibiting their extravasation. It can also undergo translocation from the plasma membrane to the nucleus, and redirect gene expression. It can simultaneously carry APC to the nucleus. (Esmon 2004a) APC is inactivated by alpha1-antitrypsin, PC inhibitor and alpha2-macroglobulin (Esmon 2000).

Hereditary PC and PS deficiency (Esmon 2000), and APC resistance (factor V Leiden) (Mosnier et al 2007) increase the risk of venous thrombosis. Low levels of PC or APC have been found in the plasma of patients with post-infection ischemic stroke (Griffin et al 2006), sepsis (Fourrier et al 1992; Lorente et al 1993), and acute lung injury (Ware et al 2003). High plasma levels of APC have been found to associate with a lower risk of ischemic stroke (Griffin et al 2006). In an experimental study, a significant decrease in PC levels was found at the onset of severe AP (Ottesen et al 1999). In experimental animal studies, infusion of APC or recombinant APC has been found to have a neuroprotective effect (Griffin et al 2006), to decrease serum markers of inflammation (Yamanel et al 2005), to increase EPCR and TM expression in pancreatic tissue in severe AP (Ping et al 2009), and to improve survival in severe AP (Alsfasser et al 2006). In a clinical study, recombinant APC (drotrecogin alfa) was found to reduce mortality in severe sepsis patients (Bernard et al 2001).

2.6.3.7 Prothrombin, prothrombin fragments F1+2 and thrombin

Prothrombin is a vitamin-K-dependent serine protease. It is produced in the liver, and at much lower levels in other tissues, such as the brain, diaphragm, kidney, spleen and intestine. (Licari and Kovacic 2009) Prothrombinase complex cleaves prothrombin to thrombin, and at the same time prothrombin fragments (F1+2) are released. F1+2 are used as an *in vivo* thrombin generation marker. Thrombin plays an essential role in both pro- and anticoagulant processes. It converts fibrinogen to fibrin, and activates FXI by generating in this way FIXa. It also activates FXIII, TAFI and other cofactors, and stimulates platelets via PARs (Dahlback 2000; Mackman 2004). Thrombin bound to TM activates PC, and in this way enhances the anticoagulant system (Esmon 2000). Thrombin is inhibited by AT and PC inhibitor (Esmon 2000).

2.6.3.8 Tissue factor and tissue factor pathway inhibitor

Tissue factor

TF is a transmembrane protein consisting of three domains: an extracellular, transmembrane and cytoplasmic domain (Spicer et al 1987). The extracellular and transmembrane domains take part in forming complex with FVIIa, and in activating FVII, FIX and FX (Martin et al 1995; Kittur et al 2004). Additionally, the cytoplasmic domain is involved in signal transduction (Ott et al 2005). TF is expressed in the vascular adventitia, in organ capsules, in myocardial cells (Drake et al 1989; Fleck et al 1990) and astrocytes (Eddleston et al 1993). It is found in epidermis, lungs, placenta and in the central nervous system (Drake et al 1989; Fleck et al 1990). Macrophages and monocyte express TF after inflammatory cytokine stimulation (Esmon 1999; Nijziel et al 2001; Bouchard and Tracy 2003) and tumor cells express it (Rak et al 2009). There are controversial reports suggesting that also platelets and granulocytes might express TF when resting or after stimulation (Eilertsen and Osterud 2004; Butenas et al 2009), and that microparticles derived from these cells might bear TF (Furie and Furie 2007). A soluble form of TF (spliced TF) circulating in blood has also been identified (Bogdanov et al 2003). The TF-factor VIIa complex has two plasma inhibitors: TFPI and AT (Morrissey 2001).

Normally, in a healthy person, physiologically active TF is not expressed by cells that are in contact with blood (Butenas and Mann 2004). After damage to the endothelial wall, however, subendothelial TF is exposed to blood and can bind plasma factor VIIa, forming TF-factor VIIa complex. This complex is known to initiate blood coagulation by activating factors IX and X, leading ultimately to thrombin generation and fibrin formation. The role of soluble TF is still unknown, and there are controversial findings regarding its procoagulant qualities (Eilertsen and Osterud 2004; Butenas et al 2009). TF is also thought to be a central link between the coagulation and inflammation pathways, and this function is mainly performed through the PARs (1-4) (Pawlinski and Mackman 2004).

High levels of TF have been found in plasma from patients with sepsis, trauma (Gando et al 1998), acute coronary syndromes (Soejima et al 1996; Suefuji et al 1997) and DIC (Shimura et al 1997). In many malignancies, TF has been proved to influence metastasis, tumor growth and angiogenesis (Rak et al 2006; Rak et al 2009). There are also animal and human studies indicating that TF is a therapeutic target, mainly by different inhibitors (Eilertsen and Osterud 2004).

In one study, the TF levels of AP patients were significantly higher than those of healthy volunteers, but there was no statistically significant difference in the TF levels between the severity groups of patients (Sawa et al 2006).

Tissue factor pathway inhibitor

TFPI is a plasma serine protease inhibitor and consists of three Kunitz-type domains (Girard et al 1989). The first domain binds to FVIIa, the second one binds to and inhibits FXa (Girard et al 1989), and the third domain may influence the association of TFPI with lipoproteins (Broze et al 1990) and cell surfaces (Piro and Broze 2004). TFPI is produced by the liver, monocytes, macrophages and endothelial cells (Lwaleed and Bass 2006). Intravascular TFPI is found in three different pools: the first one is in the circulation, the second is found in the cytoplasm of platelets, and the third, and largest, pool is bound to the endothelium (DelGiudice and White 2009). TFPI exits in plasma both as a full-length molecule, and variably as carboxy-terminal truncated forms (Warshawsky et al 1995). In plasma, approximately 80% of TFPI circulates in complex with lipoproteins (Lwaleed and Bass 2006) and about 5-20% of TFPI circulates free (Lindahl et al 1992) containing both full-length and C-terminal truncated forms (Broze et al 1994). Heparin and glycosaminoglycans induce free TFPI released from the vessel wall (Sandset et al 1988).

TFPI binds and inhibits factor Xa and TF-factor VII/VIIa complex. The full-length TFPI molecule is more efficient in inhibiting TF-FVIIa activity than the C-terminal truncated form (Warshawsky et al 1995). The free TFPI has a more potent anticoagulant effect than the lipoprotein-associated TFPI (Lindahl et al 1992).

The role of TF and TFPI has been studied in various models of sepsis. In most of these studies the main aim has been to inhibit TF, for example by antibodies or by using recombinant TFPI (rTFPI). There are also human studies in which rTFPI has been used with controversial results, regarding mortality and side effects (Pawlinski and Mackman 2004; DelGiudice and White 2009). High levels of TFPI have been found in the plasma of patients with deep venous thrombosis (Sidelmann et al 2008), acute myocardial infarction (Brodin et al 2004), hyperthyroidism (Ozcan et al 2003), pregnancy hypertension (Abdel Gader et al 2006), and high factor XI-TFPI complex levels in patients with solid malignant tumors (Iversen et al 2002). Low plasma levels of TFPI have been found to increase the risk of venous thrombosis (Dahm et al 2003).

One study has investigated the TFPI levels in AP patients (Yasuda et al 2009). The levels were significantly higher than those in healthy volunteers, and there was a significant difference in the TFPI levels between the severity groups of the patients. However, there was no significant difference in the TFPI levels between the survivors and non-survivors.

2.6.3.9 Platelets

In the thrombus formation, the platelet plug intertwines with the fibrin meshwork (Furie and Furie 2007), and the initial adhesion of platelets at the sites of vascular injury is mediated by von Willebrand factor (VWF) and fibrinogen (Furie and Furie 2007; Lippi et al 2009). They have an active and necessary role in the amplification and propagation phases of coagulation, as they provide a localizing surface in the direct proximity of the damaged area, in which most of the elements needed for coagulation are situated. In addition they provide a negatively charged phospholipid surface on which reactions can occur. (Esmon 1999; Furie and Furie 2007) Platelets are activated by exposed collagen after vascular damage, and by thrombin generated at the site of vascular damage ((Furie and Furie 2007). When activated, they are able to generate ROSs, NO and proinflammatory mediators (Uhlmann et al 2008).

In clinical studies, platelets have been shown to be activated, their indices have been elevated, and functional changes have occurred in AP (Kakafika et al 2007). In patients with severe AP, low plasma levels of platelets were found, and they were prognostic for poor outcome (Maeda et al 2006).

2.6.3.10 Protein S

PS is a plasma glycoprotein circulating in two forms: a free form (40%) and a complex form bound to complement protein C4b binding protein (60%). Only the free form retains anticoagulant activity. (Taylor et al 1991) In the anticoagulant pathway, PS works as a cofactor to PC, inactivating factors Va and VIIIa. (Lippi et al 2009)

2.6.3.11 Antithrombin

AT is a plasma serine protease inhibitor and a glycoprotein which consists of three beta-sheets, nine alpha-helices and a reactive centre loop. It exists in two isoforms, alpha (90%) and beta (10%). (Roemisch et al 2002; Quinsey et al 2004) The beta-isoform appears to be

enriched in the vessel wall (Roemisch et al 2002) and has greater affinity for heparin (Wiedermann 2006). AT is synthesized in the liver (Quinsey et al 2004).

AT is a natural anticoagulant and has also anti-inflammatory properties. It binds irreversibly and primarily to factors Xa, IXa and thrombin. However, it has also minor inhibitory effects on factors XIIa, XIa, and the TF-factor VIIa complex (Lippi et al 2009). (Esmon 2004a) AT inhibits also plasmin and trypsin (Abildgaard 2007). The anticoagulant activity of AT is significantly enhanced by heparin (Esmon 2004a; Tanaka et al 2009) and vessel wall-associated glycosaminoglycans (GAG) (Roemisch et al 2002). The anti-inflammatory properties of AT can be either indirect or direct. The indirect effects are induced by AT's anticoagulant actions; by binding thrombin or factor Xa, AT can block their proinflammatory actions at the same time. AT has direct anti-inflammatory properties by inducing prostacyclin release from endothelial cells, by reducing leukocyte-endothelium interactions (leukocyte rolling and adhesion), by inhibiting cellular signaling and by modulating cellular receptor expression (Mammen 1998; Esmon 2005b). These actions are probably mediated by the binding of AT to cell-surface GAGs and syndecan-4. Heparin is known to block the anti-inflammatory actions of AT by preventing its binding to GAGs. (Wiedermann and Romisch 2002; Roemisch et al 2002)

Congenital AT deficiencies (both quantitative and qualitative) increase the risk for venous thrombosis (Maclean and Tait 2007). Low plasma levels of AT have been found in patients with sepsis (Fourrier et al 1992; Lorente et al 1993; Roemisch et al 2002; Wiedermann 2006) and severe AP (Maeda et al 2006). In patients with severe AP, low AT levels (cutoff value < 69%) at admission were found to predict a poor outcome (Maeda et al 2006). In an experimental study, treatment with AT was shown to improve survival in AP (Bleeker et al 1992), to prevent cerulein-induced AP, and to inhibit secretion of cytokines and HMGB1 (Hagiwara et al 2009). However, infusion of AT in patients with sepsis has not been shown to reduce mortality in a clinical setting (Afshari et al 2007; Levi et al 2008). Opposite results have also been reported in experimental and small clinical studies (Roemisch et al 2002; Wiedermann 2006).

2.6.3.12 Fibrin and plasminogen-plasmin system

The production of fibrinogen is increased in inflammation, and fibrinogen and fibrin increase the production of proinflammatory cytokines and chemokines (Esmon 2005b). Fibrinolysis is essential for removing the formed clot, and it is mediated by plasmin. Plasmin cleaves fibrin,

resulting in the production of fibrin degradation products (D-dimer) (Adams and Bird 2009). Plasmin is produced as a result of proteolytic cleavage of plasminogen by tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). T-PA and u-PA are regulated by PAI. In inflammation, also PAI levels are increased, and fibrinolytic activity is decreased. (Esmon 2004b)

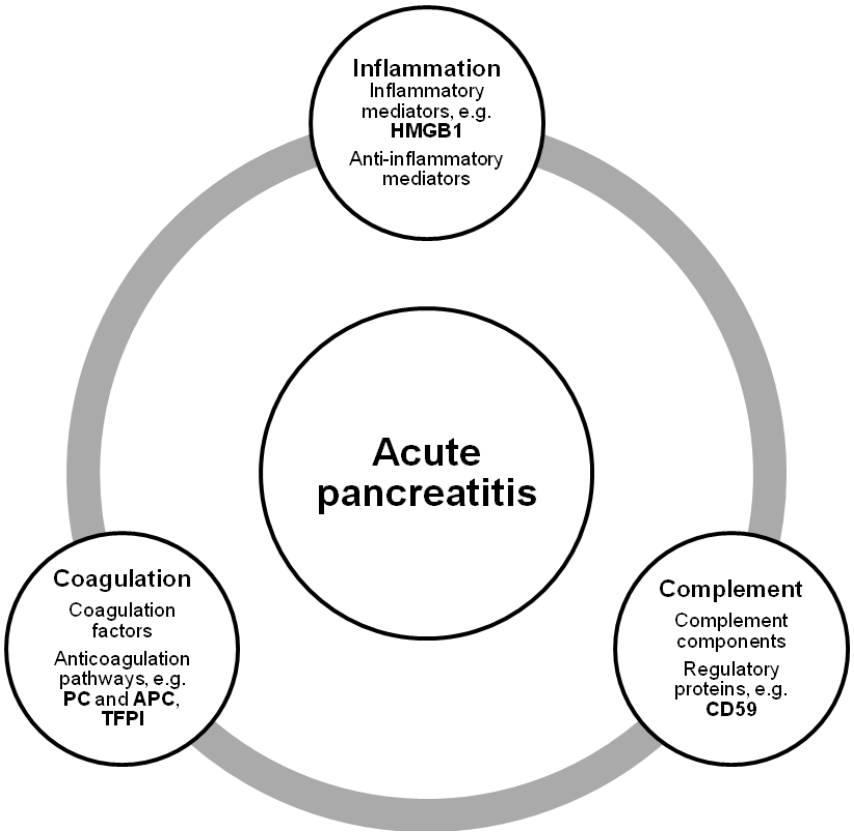

Figure 8. Acute pancreatitis is characterized by activation of inflammation, coagulation and the complement system.

2.7 Treatment of acute pancreatitis

There is no specific treatment for AP. Several pharmacologic therapies have been proposed, but none have proven to have clinical benefit in randomized controlled trials. Currently, the therapy for AP is mainly supportive. This includes adequate fluid resuscitation, monitoring for

hypoxemia and relief of pain. Nearly half of the patients with severe AP have OF and require management in the intensive care unit (Neoptolemos et al 1998; Swaroop et al 2004).

The aim of intravenous fluid resuscitation in AP is to adequately perfuse the pancreatic microcirculation, and to minimize and prevent pancreatic necrosis and its complications (Tenner 2004). Although there is widespread acceptance of the importance of aggressive fluid resuscitation in AP, only few clinical studies have been carried out in, and there are no published studies on humans, in which targeted outcome measures have been used (Gardner et al 2008). In animal studies, the benefits of fluid resuscitation (improvements in circulations and survival) (Niederau et al 1988; Juvonen et al 1999) have been demonstrated, and there is some evidence that colloid solutions might be more effective than crystalloids (Gardner et al 2008).

Infection of pancreatic necrosis is the major cause of morbidity and mortality in AP after the first 1-2 weeks of disease onset. Prophylactic antibiotic treatment has therefore been used. Several studies have nevertheless presented controversial results on the benefits of this treatment. A recent review of 7 meta-analyses, 7 reviews and some clinical studies concluded that antibiotic prophylaxis should not be used in patients with necrotizing pancreatitis. Instead, a more measured, on-demand use of antibiotics was recommended, and antibiotics should be added if signs and symptoms of infection are present (e.g. fever, leukocytosis, positive results of cultures, hemodynamic instability or OF). (Segarra-Newnham and Hough 2009)

Currently, enteral feeding (with a nasogastric or nasojejunal tube) is considered to be the preferred method of delivering nutrition in severe AP, instead of total parenteral nutrition (TPN). Two meta-analyses of randomized trials found that patients with enteral nutrition had an overall reduction in infections and in the need for pancreatic surgery, and a lower mortality rate than did patients with TPN (Marik and Zaloga 2004; Petrov et al 2008).

Patients with mild AP and sterile pancreatic necrosis should be treated conservatively. The development of infected pancreatic necrosis is an indication for intervention, usually surgical. However, early surgery (within the first 2 weeks) should be avoided, if possible because it is associated with increased mortality (Whitcomb 2006; Tonsi et al 2009). (Banks et al 2006; Forsmark et al 2007) If abdominal compartment syndrome is recognized, prompt treatment by decompressive laparotomy might be needed (Leppaniemi et al 2007). ERCP is recommended in the management of acute biliary pancreatitis, if clinical or radiographic findings suggest obstruction, e.g. due to a persistent common bile duct stone, or when acute

cholangitis is present. Cholecystectomy is also recommended in 2-4 weeks after discharge to prevent relapses. If the patient is not fit for surgery, an endoscopic sphincterotomy is a reasonable alternative (Alexakis and Neoptolemos 2005). (Beger and Rau 2007; Forsmark et al 2007; Pandol et al 2007)

2.8 Prognosis of acute pancreatitis

10% of severe AP patients who survive the initial episode die within a few years due to alcoholism and pancreas-related disease, mainly diabetes. The long-term health-related quality of life (HRQL) of the survivors is similar to that of the normal population. (Halonen et al 2003) However, in the first year after necrotizing AP, the patients show improvement in their physiological components of quality of life and their physical function, but their outcome at one year is still poor compared to the general population (Wright et al 2009). About one half of the patients with a first attack of alcohol-related AP will develop a recurrent attack. A mild first attack is also a risk factor for multiple recurrences. Continuous alcohol consumption is a risk factor for overall recurrence. (Pelli et al 2008) After follow-up, 30% of patients with alcohol-induced severe AP had remained abstinent, and 28% were problem drinkers, alcohol-dependent or alcoholics; 87% of all severe AP patients had returned to work (Halonen et al 2003). After the first episode of alcohol AP, about one third of the patients developed impaired glucose metabolism, but exocrine dysfunction did not persist during the follow-up (Pelli et al 2009). The proportion of patients with chronic changes detected by secretin-stimulated magnetic resonance pancreatography (SMRP) increased from one fifth to one half, regardless of continuing alcohol consumption during the follow-up, and chronic pseudocysts detected by SMRP were found to be a risk factor for recurrent pancreatitis (Pelli et al 2009).

3 PRESENT INVESTIGATION

3.1 Aims of the study

The aim of the present study was to investigate inflammatory and coagulation disturbances in AP. The specific aims were:

1. To study how the PC pathway evolves during the course of severe AP, and to assess the plasma thrombin formation capacity in patients with AP; to determine whether a failure of the PC pathway homeostasis is associated with the development of OF, and to find out whether the levels and functions of individual coagulation factors are associated with the severity and fatal outcome in AP patients.

2. To study the serum levels of CD59, and the plasma levels of sRAGE and HMGB1 in patients with AP; to examine how these levels are related to the development of OF and mortality in patients with AP.

3. To find inflammatory or coagulation markers for predicting the development of OF or fatal outcome in AP.

3.2 Materials and Methods

3.2.1 Patients

The Ethics Committee of the Helsinki University Central Hospital approved the study protocols, and the informed consent of each patient was obtained. All of the patients studied had AP and were admitted to Helsinki University Central Hospital within 72 h of the onset of symptoms. During the entire study period (between August 1998 - June 2004) the Pancreas Study Group of the University of Helsinki collected prospectively AP patients, and the patient series (I-IV) were chosen from these patients. Every patient series (I-IV) was comprised of different patients.

I) The study population consisted of 31 patients with severe AP treated in the intensive care unit between April 2001 and February 2003. OF developed in 13 patients (Grade 2); the remaining 18 patients without OF (Grade 1) served as controls (**Table 3**).

Table 3. Characteristics of the patients (Study I)

	Grade 1 (n=18)	Grade 2 (n=13)
Age *	44 (24-75)	47 (31-83)
Gender (female/male)#	7/11	1/12
Etiology		
Alcohol #	15	11
Gallstone #	3	2
Mortality #	0	3

* Years, median (range)

Number of patients

II) The study comprised 12 AP patients with OF and 27 control AP patients admitted to the hospital between August 1998 and July 2000. For each of the 12 OF patients (Grade 2) two to three age-matched (+/- 10 years) control patients were included. Of these patients, 13 had mild AP (Grade 0) and 14 severe AP without OF (Grade 1) (**Table 4**).

Table 4. Characteristics of the patients (Study II)

	Grade 0 (n=13)	Grade 1 (n=14)	Grade 2 (n=12)
Age *	43 (28-77)	52 (32-81)	50 (27-74)
Gender (female/male)#	5/8	2/12	2/10
Etiology			
Alcohol #	10	10	10
Gallstone #	1	3	2
Unknown #	2	1	0
Mortality #	0	0	4

* Years, median (range)

Number of patients

III) The study included 455 AP patients admitted to the hospital between September 1998 and May 2004. 38 patients had OF (Grade 2), and a control group of 127 patients was randomly selected from the 417 patients without OF. In the control group, 89 patients had mild AP (Grade 0) and 38 severe AP without OF (Grade 1) (**Table 5**).

Table 5. Characteristics of the patients (Study III)

	Grade 0 (n=89)	Grade 1 (n=38)	Grade 2 (n=38)
Age *	47 (19-87)	51 (20-79)	48 (27-88)
Gender (female/male)#	38/51	10/28	7/31
Etiology			
Alcohol #	55	29	31
Gallstone #	21	5	5
Unknown #	13	4	2
Mortality #	0	0	11

* Years, median (range)

Number of patients

IV) The study comprised 9 AP patients with OF (Grade 2) and 24 control AP patients admitted to the hospital between September 2001 and June 2004. For each of the 9 OF patients, one to two age-matched (+/- 10 years) control patients were included. Of these patients, 11 had mild AP (Grade 0) and 13 severe AP without OF (Grade 1) (**Table 6**). Also the thrombograms of 9 healthy individuals were analyzed.

Table 6. Characteristics of the patients (Study IV)

	Grade 0 (n=11)	Grade 1 (n=13)	Grade 2 (n=9)
Age *	46 (26-83)	46 (36-92)	46 (27-83)
Gender (female/male)#	4/7	3/10	2/7
Etiology			
Alcohol #	7	10	7
Gallstone #	2	3	1
Unknown #	2	0	1
Mortality #	0	0	4

* Years, median (range)

Number of patients

3.2.2 Diagnosis and Classification

The diagnosis of AP was based on typical clinical findings, including onset of epigastric pain, nausea and vomiting, and an elevated serum or plasma amylase concentration of at least 2- or 3-fold the upper reference limit, and/or typical radiological appearance of AP on CT scan. The included patients were retrospectively categorized into those with mild AP or severe AP according to the Atlanta classification (Bradley 1993). The patients with severe AP were

further subcategorized into those with local complications only (including necrosis, an abscess or a pseudocyst), and recovering without OF, and into those with OF. OF was defined as a development of respiratory failure necessitating mechanical ventilation, and/or renal failure necessitating hemodialysis. The criteria for initiating mechanical ventilation were tachypnea (respiratory rate >35/min) and/or the need for an inspiratory oxygen fraction >0.6 in order to maintain arterial partial pressure of oxygen >8 kPa. Hemodialysis was started in patients with significantly impaired renal function as indicated by increased concentrations of serum creatinine (>300 umol/l) and serum urea (>40 mmol/l) and progressive metabolic acidosis (pH <7.28) in serial measurements, regardless of urine output.

3.2.3 Scoring systems

Two scoring systems were used to describe the patients' clinical condition. Appropriate physiological and laboratory data were collected from patient records to calculate APACHE II score (II-IV) and SOFA score (II).

3.2.4 Analytical Methods

3.2.4.1 Blood samples

I) Blood samples for determining PC, APC and flow cytometry were taken every second day during the patient's stay in the intensive care unit (**Table 7**). The samples were anticoagulated. Immediately after withdrawal, the blood samples for flow cytometry were cooled in an ice-cold water bath, and kept at 0°C until processed within 24 h. From the rest of the sample, plasma was separated by centrifugation at +4°C and stored in aliquots at -70°C.

Samples on admission (defined as sampling within 36 h of actual admission) were available from 11 Grade 2 patients and 15 Grade 1 patients. OF developed in -2-14 days (median, 1 day) after admission to the research hospital. Samples preceding the diagnosis of OF were available from only five patients. Follow-up samples with a maximum 2-day interval for 10 days were available from nine Grade 2 patients.

Table 7. Collection of blood samples (Study I)

Time (day)	Every second day *, starting from day 1

* Every second day during the patient's stay in the intensive care unit; length of the stay in the intensive care unit, median (range): in Grade 2 patients 26 days (2-129) and in Grade 1 patients 6 days (2-20)

II) Serum samples for determining CD59 were collected on admission, and on days 1 and 3-7 post admission (**Table 8**). After coagulation and centrifugation, the samples were frozen and stored at -70°C.

Table 8. Collection of serum samples (Study II)

Time (day)	On admission	1	3-7 *

* The first available sample for the time period was used

III) Plasma samples for determining sRAGE and HMGB1 were collected on admission, and on days 1 and 2, days 3 and 4, and days 7 and 12 after admission (**Table 9**). After anticoagulation and centrifugation, the samples were frozen and stored at -70°C.

For determining sRAGE, samples were available on admission from 79 Grade 0, 38 Grade 1 and 34 Grade 2 patients. On days 1 and 2, these samples were available from 78 Grade 0, 28 Grade 1 and 29 Grade 2 patients. On days 3 and 4, samples were available from 55 Grade 0, 24 Grade 1 and 23 Grade 2 patients. On days 7 and 12, samples were available from 10 Grade 0, 17 Grade 1 and 25 Grade 2 patients.

For determining HMGB1, samples were available on admission from 67 Grade 0, 35 Grade 1 and 29 Grade 2 patients. On days 1 and 2, these samples were available from 73 Grade 0, 24 Grade 1 and 26 Grade 2 patients. On days 3 and 4, samples were available from 45 Grade 0, 19 Grade 1 and 25 Grade 2 patients. On days 7 and 12, samples were available from 9 Grade 0, 17 Grade 1 and 23 Grade 2 patients.

Table 9. Collection of plasma samples (Study III)

Time (day)	On admission	1-2 *	3-4 *	7-12 *

* The first available sample for each time period was used

IV) Plasma samples for determining F1+2, TFPI (free and total) and thrombogram were collected on admission (**Table 10**). After anticoagulation and centrifugation, the samples were frozen and stored at -70°C.

Table 10. Collection of plasma samples (Study IV)

Time (day)	On admission

3.2.4.2 Laboratory tests

The plasma levels of PC and APC were determined by enzyme capture assay in the laboratory of Professor J.H. Griffin, Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, USA (I). Briefly, a monoclonal antibody against PC and APC was immobilized in microplates. Plasma samples supplemented with benzamidine, a reversible inhibitor of thrombin, APC, and trypsin-like proteases, were incubated in the wells for the capture of APC and PC. The plates were then washed to remove sample constituents and benzamidine. The amidolytic activity of the captured APC was measured with chromogenic substrate S-2366 (Chromogenix AB, Mölndal, Sweden). Assays were run in duplicate, and a noncommercial plasma pool containing benzamidine was used as a standard. The total PC was measured by activating the bound PC in the wells by Protac (American Diagnostica, Greenwich, CT, USA). The amidolytic activity was then measured with the chromogenic substrate S-2366 (Chromogenix AB). The amidolytic activity observed after Protac activation is essentially equal to the total PC. Assays were run in duplicate.

CD59 levels were measured by means of a sandwich enzyme immunoassay in the laboratory of Professor S. Meri, Haartman Institute, University of Helsinki, Finland (II). Rat mAb YTH53.1 against human CD59 was originally provided by Professor H. Waldmann (Sir William Dunn School of Pathology, Oxford, UK). Nunc Maxisorp microtiter plates were coated with YTH53.1 (5 ug/ml in 0.1 M $NaHCO_3$, pH 8.5) overnight at +4°C. The wells were washed with phosphate-buffered saline (PBS)/0.02% Tween 20, and the samples, diluted 1:20 in washing buffer, were added. CD59 was purified from urine as described previously (Lehto et al 1995) and used as standard at concentrations of 0-40 ng/ml. The samples were incubated for 1 h at room temperature, the wells were washed and the primary antibody goat-anti-human CD59 (a gift from Professor F. Tedesco, Department of Physiology and Pathology, University of Trieste, Trieste, Italy) diluted 1:2000 in PBS/Tween was added. After 1 h of incubation, the plates were washed and horseradish peroxidase (HRP)-conjugated rabbit-anti-goat antibody (Dakopatts, Denmark) diluted 1:5000 in PBS/Tween with 1% rat serum was added. The plates were incubated for 1 h at RT, washed with PBS/Tween and the OPD

substrate (Dakopatts) was added. The reaction was stopped with 2 M H_2SO_4 and colour formation was measured with a spectrophotometer.

The levels of sRAGE in the plasma samples were measured by DuoSet human RAGE enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, Minn, USA) in the laboratory of Professor H. Rauvala, Neuroscience Center, University of Helsinki, Finland (III).

Plasma HMGB1 levels were measured by combined heparin-Sepharose precipitation and Western blotting in the laboratory of Professor H. Rauvala, Neuroscience Center, University of Helsinki, Finland (III). Briefly, the plasma samples were centrifuged at 14,000-15,000*g* for 2 min, and 120 uL of supernatant were collected. 60 uL of 50% heparin-Sepharose slurry in phosphate-buffered saline containing 1.2 mol/L NaCl were added to the supernatants. The samples were mixed by rotation at 4°C for 1-2 h, after which the supernatant was removed and the gel washed with phosphate-buffered saline. Heparin-Sepharose-bound proteins were eluted with 30 uL of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer by incubating at 99°C for 5 min in a shaker. 15 uL of the samples were electrophoresed in SDS-PAGE and analyzed in Western blot gels probed with chicken anti-HMGB1 immunoglobulin Y^{32} and peroxidase conjugated anti-chicken antibodies (Zymed Laboratories, San Francisco, CA, USA). Normal plasma samples containing known amounts of recombinant HMGB1 were analyzed equally in each gel, and a standard curve was generated. Recombinant HMGB1 protein was produced as described (Parkkinen et al 1993), and the protein concentration was determined by measuring absorbance at 280 nm, whereas protein purity was determined by SDS-PAGE analysis. A standard curve was accomplished by adding a known amount of recombinant HMGB1 to normal human plasma, which is virtually free of HMGB1 (Rouhiainen et al 2000). Optical densities of the immunoreactive 30-kD bands were quantified as described (Rouhiainen et al 2004; Sunden-Cullberg et al 2006).

The F1+2 levels in the plasma samples were measured by Enzygnost* F1+2 micro ELISA (Dade Behring Inc., Marburg, Germany) (IV). The TFPI (free and total) levels in the plasma samples were measured by Asserachrom^R free and total TFPI ELISA (Diagnostica Stago, Asnieres-Sur-Seine, France) (IV). These measurements were performed in the Haematology Laboratory, Helsinki University Central Hospital, Finland.

Values for routine coagulation parameters (PT, D-dimer, and platelet count) (I), (PT, APTT, and platelet count) (IV) and CRP (III, IV) were determined in accordance with the hospital routine laboratory practice.

3.2.4.3 Flow cytometry (I)

APC acts *in vitro* as an anti-inflammatory agent mainly by modulating monocyte activation during inflammation (Grey et al 1994; White et al 2000; Yuksel et al 2002). Monocyte HLA-DR expression reflects the recent history of activation/functional suppression of the circulating monocyte population (Kylanpaa-Back et al 2001). HLA-DR was determined with cytometry of whole blood flow, as described previously (Kylanpaa-Back et al 2001; Mentula et al 2003). The determinations were done at the Department of Bacteriology and Immunology, Haartman Institute, University of Helsinki, Finland. The mAbs were phycoerythrin-conjugated anti-HLA-DR mAb (IgG2a, clone L243), phycoerythrin-conjugated irrelevant mAb (IgG2a, clone X39), and fluorescein isothiocyanate-conjugated anti-CD14 mAb (IgG2b, clone MFP9) (Becton Dickinson, San Jose, CA, USA). HLA-DR expression is defined as the proportion of positively fluorescing monocytes (HLA-DR%).

3.2.4.4 Thrombogram (IV)

The thrombograms were obtained through Calibrated Automated Thrombography (CAT) in the Haematology Laboratory of Helsinki University Central Hospital, Finland. The parameters of the thrombogram are: lag time, area under the curve (AUC=endogenous thrombin potential ETP), peak height, and time to reach the peak (Hemker et al 2002). Platelet-poor plasma (PPP) was used. Coagulation was triggered by recalcification in the presence of 5 pM TF. Experiments were carried out in the absence or in the presence of APC at various concentrations (1 nM and 2.5 nM).

3.2.5 Statistical Methods

The results were expressed as medians and ranges or inter-quartile ranges (IQR). Comparisons between two groups were done by the Mann-Whitney U test (I-IV). For comparing three groups, the Kruskall-Wallis test (III, IV) and the Jonkheere-Terpstra test for trend (IV) were used. The *P* values were corrected by the Bonferroni method (III). When the Kruskall-Wallis test result was significant, the Dunn *post hoc* test (III, IV) was used to test significance of differences between the 3 groups. Fisher's exact test (I) or Chi-square tests (I, III) were used for comparison of proportions. Comparison of the follow-up samples was done with Wilcoxon signed rank test (III) or Friedman's test (I, III), followed by Dunn's test for *post hoc* comparisons (I). Spearman's rank correlation was used for assessing correlations (I-IV). Optimal cutoff levels for values and markers were determined using receiver-operator

characteristic (ROC) curves (I, II, IV). Sensitivity, specificity and positive and negative likelihood ratios (LRs) were calculated with 95% confidence intervals (CI) according to the efficient-score method, and corrected for continuity (II). Statistical analyses were performed using the SPSS statistical software (Chicago, IL, USA). $P<0.05$ was considered statistically significant.

3.3 Results

3.3.1 Upregulated but insufficient generation of activated protein C in severe acute pancreatitis (I)

In this study there were 13 patients with severe AP and OF (Grade 2), and 18 patients with severe AP without OF (Grade 1). Grade 2 patients were compared with Grade 1 patients. Three patients died (10% of all patients) and they were Grade 2 patients (23% of Grade 2 patients).

During the observation period, decreased PC values (<70% of the adult mean) were a frequent finding, occurring in 43% of all samples (68% of all patients) at various stages of the disease. All but one of the APC values fell within the range observed in healthy resting adults. The APC/PC ratio did not fall below the lower limit of normal in any sample, but exceeded the upper normal limit in 40% of the samples. At least one APC/PC ratio value was above normal in 74% of all patients. The intrapatient PC levels varied greatly from day to day. The levels of APC showed much less variation than did the PC levels.

On admission to hospital, the APC concentration was significantly lower in Grade 2 patients than in Grade 1 patients (median APC 86% vs 105%, p=0.027), whereas the PC level and the APC/PC ratio did not differ significantly between the two groups. However, 89% of Grade 2 patients and 43% of Grade 1 patients showed an abnormally high APC/PC ratio.

During the first two weeks of hospitalization, low PC levels occurred in 92% of Grade 2 patients and 44% of Grade 1 patients (p=0.008). The minimum APC level was lower in Grade 2 patients than in Grade 1 patients (median 85% vs 97%, p=0.009) (**Table 11**). Using 87% as the cutoff value, 8/13 (62%) of Grade 2 patients and 3/18 (17%) of Grade 1 patients had reduced APC levels (p=0.021). A total of 92% Grade 2 patients and 50% of Grade 1 patients had APC/PC ratios exceeding the upper normal limit (p=0.02).

Table 11. Activated protein C and protein C levels, their ratios, and the proportion of positively fluorescing monocytes (HLA-DR%) in patients with organ failure (Grade 2) and without organ failure (Grade 1) during their stay in the intensive care unit.

Variable	Grade 2	Grade 1	p-value
Lowest APC level (%)	85 (76-102)	97 (73-136)	0.009
Lowest PC level (%)	52 (8-70)	70 (18-113)	0.03
Lowest APC/PC ratio	1.1 (0.87-13.2)	1.3 (1.0-3.8)	0.02
Highest APC/PC ratio	1.8 (1.5-13.2)	1.6 (1.2-4.1)	0.03
HLA-DR%	46 (8-82)	44 (11-84)	0.80

Data presented as median (range)

APC=activated protein C, PC=protein C

There was no difference in HLA-DR% between Grade 2 and Grade 1 patients during their stay in the intensive care unit (**Table 11**). HLA-DR-positive monocytes correlated with PC levels ($R=0.38$, $p<0.001$) and APC levels ($R=0.27$, $p<0.001$), indicating that the PC pathway was associated with systemic inflammation-triggered immune suppression.

The plasma samples of Grade 2 patients drawn before OF, and compared with those of Grade 1 patients, showed equal APC levels ($p=0.56$), low PC levels ($p=0.025$) and high APC/PC ratios ($p=0.02$). In the subgroup of patients with OF ($n=9$) during a 10-day follow-up, both the APC and the PC levels tended to increase (**Figure 9**). The median APC/PC ratio was the lowest on day 7. The apparent mechanism for the decreasing APC/PC ratio during hospitalization was a gradual improvement of PC levels, without a concomitant increase in APC values. There was thus a trend of gradual improvement of early PC pathway disturbances during the course of OF.

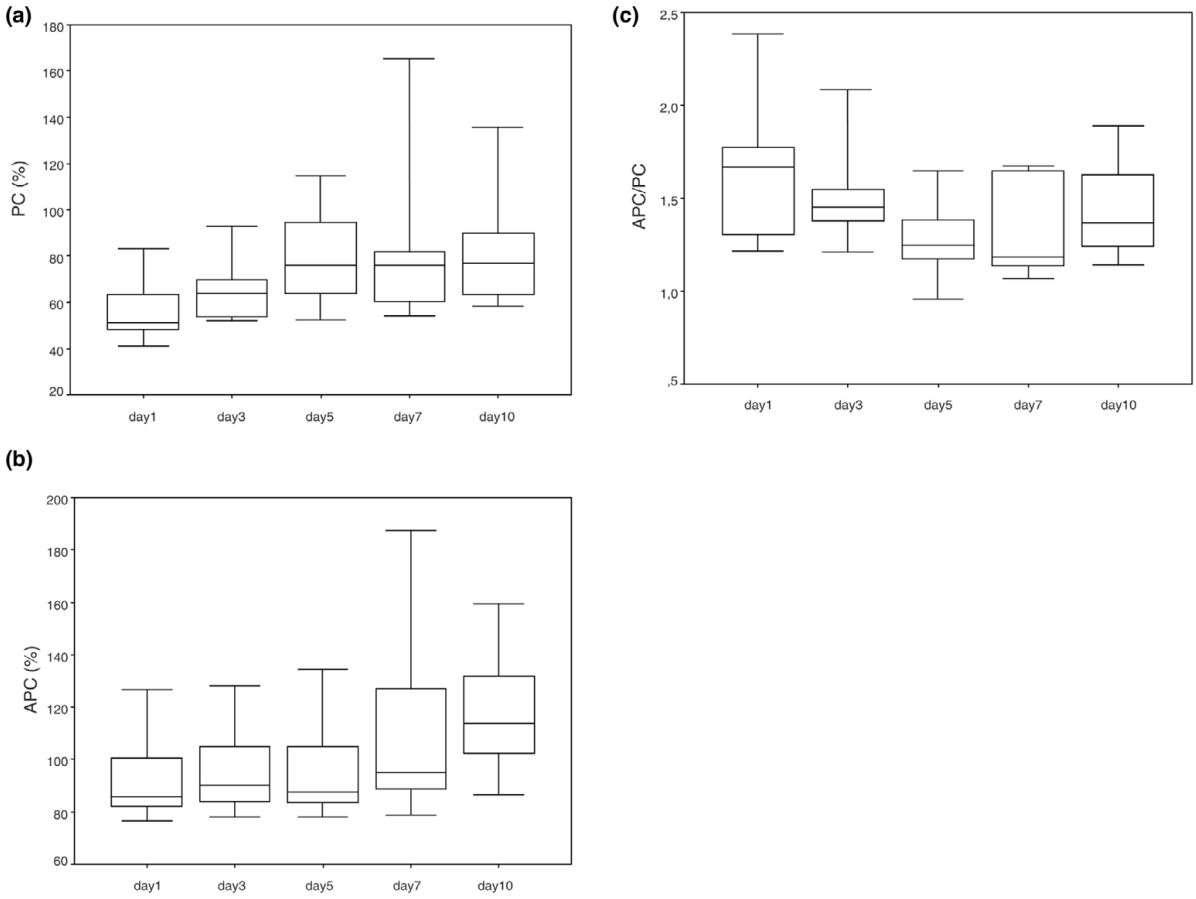

Figure 9. Follow-up concentrations of a) protein C (PC), b) activated protein C (APC), and c) APC/PC ratio during the stay in hospital in nine patients with organ failure. The time of sampling did not influence any parameter statistically significantly (Friedman's test >0.05).

In the three non-survivors, the median level of APC was 105% (85-188), that of PC was 74% (8-165), and that of the APC/PC ratio was 1.45 (1.13-13.2). In the 10 surviving Grade 2 patients, the median level of APC was 98% (76-109), that of PC was 71% (35-165), and that of the APC/PC ratio was 1.32 (0.87-2.6).

3.3.2 Disturbed tissue factor regulation in acute pancreatitis (IV)

In this study there were 9 patients with severe AP and OF (Grade 2), 13 patients with severe AP but without OF (Grade 1), and 11 patients with mild AP (Grade 0). The Grade 2 patients were compared with Grade 0 and Grade 1 patients. Four patients died (12% of all patients); they were all Grade 2 patients (44% of Grade 2 patients). For the thrombogram analyses, the thrombograms of 9 healthy individuals served as controls.

On admission, there was no significant difference in F1+2 levels between the patient groups.

The thrombograms of all patients, as compared with healthy reference subjects, were variable and disturbed in their shape (**Figure 10**). In 11 patients [7 (78%) Grade 2 patients, 4 (31%) Grade 1 patients and 0 (0%) Grade 0 patients, p=0.001] the standard TF stimulation did not trigger thrombin generation at all, and their thrombogram lines were thus 'flat'. There was a significant increase in lag time and time to peak values associated with severity of AP, but the differences in other thrombogram variables between the patient groups were not statistically significant. When the experiments were carried out in the presence of APC (1 and 2.5 nM), there were no significant differences in thrombogram variables between the patient groups.

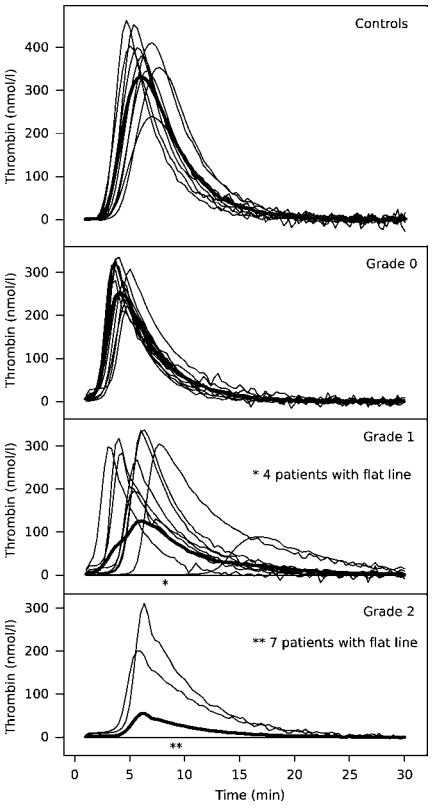

Figure 10. The thrombograms of patient groups and healthy controls. The black line represents a mean curve.

The differences between the patient groups were statistically significant at levels of free TFPI and in ratios of free and total TFPI. There was a significant rising trend in free and total TFPI levels, and in the ratios of free and total TFPI, which was associated with the severity of AP

(**Figure 11**). The differences between patients showing thrombin generation (n=22) and patients with 'flat' thrombograms (n=11) were significant in free TFPI levels and TFPI ratios, but not in total TFPI levels. The correlation between F1+2 levels and free (R=0.053, p=0.771) and total (R=0.247, p=0.166) TFPI levels and their ratios (R=-0.044, p=0.809) was not significant. In patients (n=22) whose thrombin generation started in the CAT, the correlation between total TFPI levels and lagtime values was significant (R=0.442, p=0.04), but not between free TFPI levels (R=0.376, p=0.085) or TFPI ratios (R=0.244, p=0.274) and lag time values.

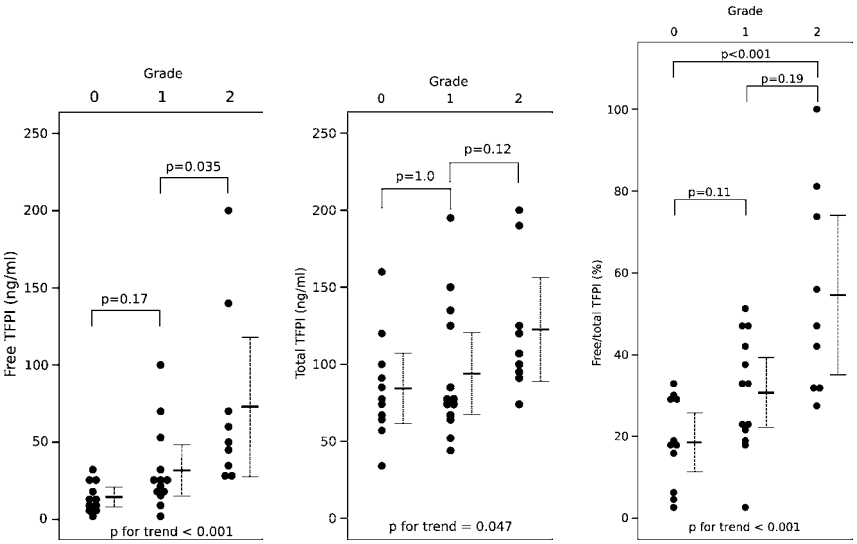

Figure 11. Dot blots showing the plasma (left) free and (middle) total tissue factor pathway inhibitor (TFPI) levels and (right) their ratios on admission. Grade 0, mild AP; Grade 1, severe acute pancreatitis, but no organ failure; and Grade 2, severe acute pancreatitis and organ failure. Error bars show the mean and 95% confidence interval of the mean for each group. Statistical significances between the groups after the Kruskall-Wallis and Dunn's *post hoc* test are shown above, and *P* values for the trend between the groups by Jonkheere-Terpstra test are shown at the bottom.

On admission, the difference in F1+2 levels between survivors (n=29) and non-survivors (n=4) was not significant. All non-survivors presented with a 'flat curve' in the CAT. The difference between survivors and non-survivors in free TFPI levels and TFPI ratios was significant, but not in total TFPI levels. Since free TFPI is evidently associated with the progressive severity of AP and the 'flat curve' in CAT, and consequently, the 'flat curve' in CAT is associated with fatal outcome, the predictive capability of these measurements was analyzed in relation to mortality. When the 'flat curve' was used on admission alone, the

sensitivity/specificity of predicting fatal outcome was 100/36%, respectively. Combining this with free TFPI (cutoff 25 ng/ml) increased the specificity to 50%.

3.3.3 Elevated levels of CD59 in severe acute pancreatitis (II)

In this study there were 12 patients with severe AP and OF (Grade 2), 14 patients with severe AP and without OF (Grade 1), and 13 patients with mild AP (Grade 0). Grade 2 patients were compared with Grade 0 and Grade 1 patients. Four patients died (10% of all patients); they were all Grade 2 patients (33% of Grade 2 patients).

On admission, the serum CD59 levels of all patients correlated significantly ($p<0.05$) with their CRP levels ($R=0.40$) and APACHE II scores ($R=0.32$). On day 1, the CD59 levels correlated significantly with the CRP levels ($R=0.37$, $p<0.05$) and APACHE II scores ($R=0.52$, $p=0.01$). A significant correlation with CRP and CD59 was seen also on days 3-7 ($R=0.38$, $p<0.05$).

On admission, the median serum CD59 levels were significantly higher ($p=0.002$) in Grade 2 (median 104.2 ng/ml, range 26.1-186.3) than in Grade 0 patients (37.3 ng/ml, range 30.3-75.9) and in Grade 1 patients (38.6 ng/ml, range 19.9-96.1). The CD59 levels remained higher in Grade 2 patients than in Grade 0 and Grade 1 patients on day 1 ($p=0.001$) and days 3-7 ($p=0.002$). The overall serum CD59 levels were higher in Grade 2 patients than in Grade 0 and Grade 1 patients ($p<0.001$). The serum CD59 levels remained stable during the observation period in Grade 0 and Grade 1 patients, and also in Grade 2 patients, except in two patients whose levels increased toward the end of the observation period.

The sensitivity, specificity, positive and negative likelihood ratios (LR) for CD59, CRP and APACHE II scores and their 95% confidence intervals (CI) in predicting OF are given in **Table 12**.

Table 12. Sensitivity, specificity, positive and negative likelihood ratio (LR) and their 95% confidence intervals (CI) in predicting organ failure in acute pancreatitis.

Time	Marker and cutoff *	Sensitivity (%) (95% CI) #	Specificity (%) (95% CI) #	Positive LR (95% CI) #	Negative LR (95% CI) #
Day 0	CD59>89	67 (35-89)	96 (79-100)	18 (2.5-128)	0.35 (0.16-0.77)
	CRP>188	67 (35-89)	81 (61-93)	3.6 (1.5-8.7)	0.41 (0.18-0.93)
	APACHE II >7	67 (35-89)	74 (53-88)	2.6 (1.2-5.5)	0.45 (0.20-1.03)

Abbreviations: CRP=C-reactive protein; APACHE=acute physiology and chronic health evaluation

* Determined using receiver-operator characteristic (ROC) curves

Calculated according to the efficient-score method and corrected for continuity

3.3.4 sRAGE and HMGB1 in patients with acute pancreatitis (III)

In this study there were 38 patients with severe AP and OF (Grade 2), 38 patients with severe AP but without OF (Grade 1), and 89 patients with mild AP (Grade 0). Grade 2 patients were compared with Grade 0 and Grade 1 patients. In addition, the Grade 0 and Grade 1 patients were combined into a single control group (Grades 0 + 1), and this group was compared with Grade 2 patients. 11 patients died (7% of all patients); they were all Grade 2 patients (29% of Grade 2 patients).

On admission and on days 1 and 2, the difference in plasma sRAGE levels was significant only between Grade 0 and Grade 1 patients. On days 3 and 4, the sRAGE level was significantly higher in Grade 2 than in Grade 0 ($p<0.05$) and Grade 1 patients ($p<0.001$). The highest measured sRAGE levels were also higher in Grade 2 than in Grade 1 patients ($p<0.01$). On days 7 and 12, there was no statistically significant difference between the 3 groups (**Figure 12**).

The median of the highest measured sRAGE levels was higher in Grade 2 patients (472 pg/ml; IQR, 259-912) than in Grades 0 + 1 patients (349 pg/ml; IQR, 209-544; $p=0.024$). Analysis of the time course of the increase in sRAGE levels in Grade 2 patients revealed a rising trend up until days 3 and 4 ($p=0.019$). Thereafter, the median sRAGE level fell, and

was lower on days 7 and 12 (253 pg/ml; IQR, 141-567; n=16) than on days 3 and 4 (536 pg/ml; IQR, 377-1112; p=0.003). In 72 of the 89 Grades 0 + 1 patients, from whom plasma samples were available at each of the 3 time points, the median sRAGE levels on day 0, days 1 and 2, and days 3 and 4 were rather similar (p=0.14).

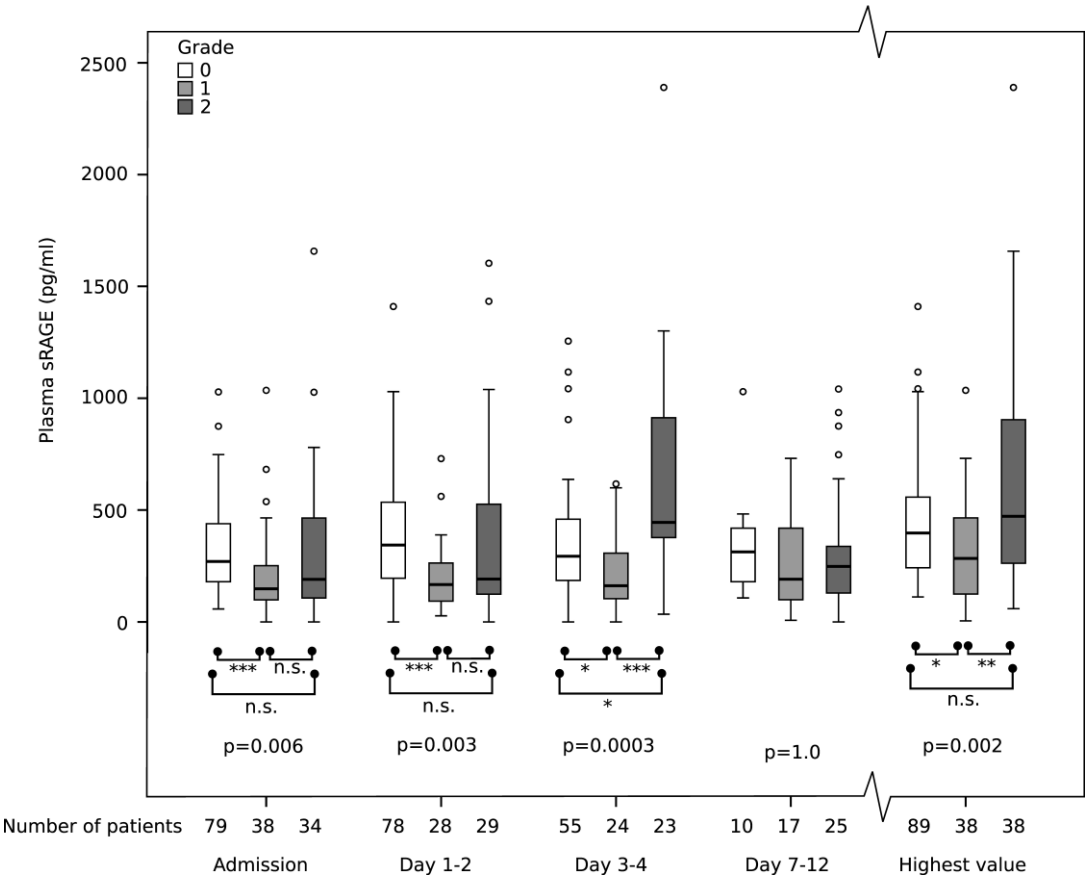

Figure 12. Box plots showing the plasma sRAGE levels (median, interquartile range, highest and lowest values, outliers) during the course of AP. Grade 0, mild AP; Grade 1, severe AP, but no organ failure; and Grade 2, severe AP and organ failure. P values for the significance of differences between the groups by Kruskall-Wallis test are shown. P values at different time points were corrected by the Bonferroni method. Post hoc test was done using Dunn's test, and the level of significance between the 2 groups is shown with asterisks (*P<0.05; **P<0.01; ***P<0.001; and n.s., not significant).

The proportion of patients with detectable HMGB1 (>30 ng/ml) did not differ significantly between the 3 groups of AP severity at any time point (p>0.05) (**Figure 13**). Among the patients with detectable HMGB1, the median values of the highest levels between the groups (Grade 2 patients vs Grades 0 + 1 patients) were similar (p=0.31).

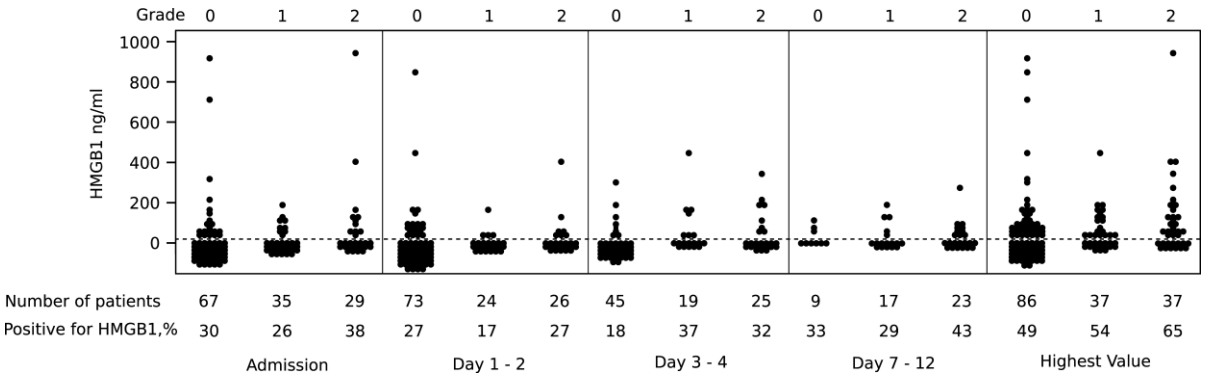

Figure 13. Dot plots showing the plasma HMGB1 levels during the course of AP. Grade 0, mild AP; Grade 1, severe AP, but no organ failure; and Grade 2, severe AP and organ failure. The dots below the broken line represent measurements of the patients with a HMGB1 level below the detection limit (30 ng/ml). The proportion of patients with detectable HMGB1 at each time point is shown below the graph; the differences between the 3 groups were not significant.

The median of the highest sRAGE levels of non-survivors (561 pg/ml; IQR, 379-1184, n=11) was higher than that of the survivors (360 pg/ml; IQR, 219-558; n=154; p=0.010). The median of the highest sRAGE levels of the non-surviving Grade 2 patients was higher than that of the Grade 2 survivors, but the difference was not significant (p=0.085). The HMGB1 of Grade 2 patients was detectable in 18 (67%) of the 27 survivors and in 6 (60%) of the 11 (55%) non-survivors (p=0.48).

3.3.5 Markers predicting organ failure and fatal outcome (I-IV)

AP patients with OF showed low PC levels, reduced APC levels and high APC/PC ratios compared with patients without OF. Plasma samples drawn immediately prior to OF showed also low PC levels and high APC/PC ratios. (I)

AP patients with OF had higher free TFPI levels, and free and total TFPI ratios compared with patients without OF. These levels were higher also in non-survivors than in survivors. All non-survivors had a ‘flat curve’ in the thrombogram. (IV)

AP patients with OF had higher CD59 levels than did patients without OF, and these levels remained higher during the first week of hospitalization. (II)

AP patients with OF had higher sRAGE levels than did patients without OF. The median of the highest sRAGE levels of non-survivors was higher than that of the survivors. (III)

3.4 Discussion

3.4.1 Coagulation disturbances in acute pancreatitis (I, IV)

In the first study we found that the minimum levels of APC were significantly lower in OF patients than in severe AP patients without OF. The PC deficiency was therefore logically associated with a decrease of APC levels in patients with OF. A more significant finding, however, may be that the APC levels did not seem to be grossly elevated. In fact, only one sample from one patient showed an absolute APC level that exceeded the reported upper limit of normality (200%) (Petaja et al 1998). Thrombin is the APC activator, and APC is a feedback inhibitor for thrombin generation (Griffin 1995; Esmon 2001). The complicated balance between thrombin, PC and APC varies depending on the circumstances. In resting healthy adults, the PC level, rather than thrombin level, may be the major determinant of the circulating APC level (Fernandez et al 1997; Petaja et al 1998; Petaja et al 1999). On the other hand, it has been reported that the most pronounced and rapid enhancements from normal resting APC levels to levels typically ranging from 250% to over 800% of the normal mean have occurred during the first minutes of reperfusion in liver transplantation and during a proinflammatory infusion in renal transplantation (Ilmakunnas et al 2003; Turunen et al 2005). In a sepsis study, it was found that despite ongoing activation of coagulation, 25% of patients failed to increase their APC levels above 250% (while 75% had levels ranging from approximately 250-800%) (Liaw et al 2004). It was concluded that the septic patients varied considerably in their ability to generate APC in response to the physiological thrombin stimulus, and the phenomenon was attributed to endothelial dysfunction (Liaw et al 2004). This is in accordance with our findings, suggesting that the lack of elevated free APC levels in the presence of activated coagulation in patients with AP in most cases reflected dysfunctional PC activation on the endothelium, or possibly enhanced inhibition of APC by plasma protease inhibitors. It is possible, however, that significant PC deficiency could also be rate-limiting for APC formation in patients with severe AP.

In the fourth study our results showed that the thrombograms of AP patients were variable and disturbed in their shape. The thrombin generation of AP patients varied greatly, and there were even 11 patients whose thrombin generation was not triggered at all by standard TF stimulation. In patients whose thrombin generation started, the severity of AP correlated significantly with the lag time values, but not with the ETP values. It thus seems that in AP the initiation of coagulation is hampered, while the thrombin generation capacity is not affected, and that the coagulation disturbances in AP are not primarily caused by the exhaustion of the coagulation factors in the plasma. This gradually hampered initiation of

coagulation is not caused by abnormally functioning APC in vitro, because the severity of AP did not correlate with lag time or ETP values in the presence of APC.

It is possible that the gradually hampered initiation of coagulation, up until it is disrupted, is caused by TFPI. In fact, this seems to be the case. There was a significant increase in free TFPI levels with the severity of AP. The total TFPI levels were higher in patients with OF than in the group of patients composed of those with mild AP plus those with severe AP but without OF. However, there was no increase in these values between patients with mild AP and severe AP without OF. This increase, especially in free TFPI levels, seems to occur in those patients whose thrombogram lines were 'flat' because their free TFPI levels and TFPI ratios were significantly higher than those of the other patients. In AP, this increase in TFPI might compensate the rise of TF caused by inflammation. It is also possible that natural heparins and endothelial damage in AP lead to the release of TFPI from endothelium.

Recombinant APC has been found to reduce the mortality rate of severe sepsis patients (Bernard et al 2001). Thus, generation and function of APC in AP are of great interest. In the first study we showed that in severe AP endogenous APC generation is upregulated but insufficient. In the fourth study we extended these findings by demonstrating that *in vitro* exogenous APC functions similarly, regardless of the severity of AP. Therefore, AP itself does not disrupt the function of APC. The value of this new information rises from the fact that acquired APC resistance might well occur in AP via disruptions in EPCR and protein C inhibitor. In experimental studies inflammatory mediators have been found to down-regulate EPCR expression (Esmon 1999), and EPCR can also be shed from endothelium by thrombin and inflammatory mediators (Esmon 2004a).

3.4.2 Inflammatory markers in acute pancreatitis (II, III)

In the second study we found that serum CD59 levels were significantly higher in patients with OF than in other AP patients. Increased plasma levels of CD59 apparently indicate the loss of CD59 from cell surfaces (Vakeva et al 1992; Rautemaa and Meri 1996). Loss of CD59 may occur in the pancreas, in blood endothelium, blood cells or in tissues, such as the lung, that are affected in AP. Why are the levels of CD59 elevated in AP? The CD59 molecule has a GPI anchor by which it binds to phospholipids on the outer leaflet of cell membranes. Thus, CD59 might well be shed off by phospholipases (Morgan 1989; Vakeva et al 1992; Rautemaa and Meri 1996) e.g. by phosphatidylinositol-specific phospholipase D (PIPLD). Many phospholipases C (PIPLC) can also cleave the GPI anchor of CD59, but this dependens on

the type of anchorage and number of phospholipids on the CD59 molecule. Only a proportion of CD59 molecules are sensitive to PIPLC enzymes. In AP, phospholipases are released, and also membrane damage may occur. Loss of membrane integrity (and e.g. phospholipase A2) might lead to the release of cell membrane vesicles containing CD59. The CD59 molecule can be cleaved by trypsin only when in reduced form, as it is generally relatively resistant to proteolytic enzymes (Meri et al 1996). Increased CD59 levels have been found also in humans during acute myocardial infarction, and myocardial damage has been found to lead to the release of CD59 from the sarcolemmal cell membrane (Vakeva et al 2000). A similar, yet more extensive and widespread process may thus be occurring in AP. Loss of CD59 is likely to predispose the cells to complement membrane damage and to inflammatory changes which the forming MAC complexes can induce. A tendency for increased complement activation has been observed in AP (Foulis et al 1982; Whicher et al 1982; Duchateau et al 1984; Roxvall et al 1989; Roxvall et al 1990) and is very likely due to the release of proteolytic enzymes from the pancreatic acinar cells.

In the third study we found that the highest measured sRAGE level during the follow-up period was significantly higher in AP patients with OF than in AP patients who recovered without developing OF. Also the non-survivors had significantly higher maximum sRAGE levels than the survivors. RAGE is expressed on endothelium, neurons, vascular smooth muscle cells, and mononuclear phagocytes (Abeyama et al 2005; Li et al 2006). The circulating pool of sRAGE consists of esRAGE and proteolytically cleaved forms of RAGE (Hudson et al 2005). RAGE and sRAGE have been studied mostly in chronic diseases (Emanuele et al 2005; Falcone et al 2005; Pullerits et al 2005; Basta et al 2006) but little is still known about their function in patients. However, it seems that high levels of sRAGE (total pool) in severe AP could be caused by damage to the endothelium, or they could be released by activated cells. High levels of sRAGE have also been found in patients with sepsis (Bopp et al 2008) and acute lung injury (Uchida et al 2006). High amniotic fluid concentrations of sRAGE and esRAGE have been shown to be associated with intra-amniotic infection or inflammation (Romero et al 2008), and administration of recombinant sRAGE has been found to reduce inflammatory responses (Wear-Maggitti et al 2004; Andrassy et al 2006; Bucciarelli et al 2006; Raman et al 2006).

In the third study we also found that the HMGB1 levels of three different severity groups of AP patients were similar. This is in agreement with the study by Yasuda and colleagues (Yasuda et al 2006) who used a commercially available ELISA kit to determine the on-admission serum HMGB1 levels. They found that the levels were higher in patients with organ dysfunction than in those without organ dysfunction, but the difference was not

significant. Furthermore, the HMGB1 levels in patients with pancreatic necrosis were similar to those in patients without pancreatic necrosis, and the levels in non-survivors were similar to those of survivors. Finally, the serum HMGB1 levels did not correlate with the Ranson score or APACHE II score. In this context it is noteworthy that the HMGB1 levels in systemic inflammation-related acute lung injury (Ueno et al 2004) determined by the Western immunoblotting method, were similar between survivors and non-survivors, and in the study on sepsis (Sunden-Cullberg et al 2006) no predictable correlation was found between the serum HMGB1 levels and the severity of sepsis. Although it is presently unclear how greatly the differences between the methods and between the specificities of the antibodies used affect the data (Sunden-Cullberg et al 2006), collectively, the above results support the view that HMGB1 levels in patients with AP, sepsis or acute lung injury neither correlate with the severity of the insult nor with the patients' outcome. Three recent studies have compared different methods of analyzing HMGB1, and they have demonstrated a strong correlation between ELISA and the Western immunoblotting method in analyzing patient plasma (Sunden-Cullberg et al 2006; Ilmakunnas et al 2008; Karlsson et al 2008). HMGB1 may nevertheless play a role in the pathogenesis of systemic inflammation-related organ dysfunction.

The cellular release of HMGB1 is associated with the post-translational modifications of the protein. Thus, HMGB1 molecules derived from necrotic cells and activated phagocytes are hypo-acetylated and hyper-acetylated, respectively. The two forms are considered to be molecularly distinct, and may differ in their inflammation-promoting capacity. (Bonaldi et al 2003; Youn and Shin 2006) The administration of HMGB1 antagonists is beneficial in experimental models of sepsis and systemic inflammation (Wang et al 2004b; Chen et al 2005; Lin et al 2005). Sawa and colleagues (Sawa et al 2006) used anti-HMGB1 neutralizing antibody in experimental AP, and found that it inhibited the elevation of serum amylase, alanine aminotransferase and creatinine, and decreased the histological alterations in the pancreas and the lung.

3.4.3 Endothelial damage, coagulation and inflammation in acute pancreatitis (I-IV)

In the pathogenesis of AP, the acinar cell injury causes leakage of pancreatic enzymes into pancreatic tissue, where they become activated and initiate autodigestion of the pancreas. The activated proteases break down tissue and cell membranes, causing edema, vascular damage, hemorrhage, necrosis and local inflammation. The microvascular derangement

propagates this event further. PC and APC can bind to EPCR, which enhances their activity. This APC-EPCR complex does not appear to display anticoagulant activity. EPCR can be shed from endothelium by thrombin and inflammatory mediators. (Esmon 2004a) CD59 can be shed from cells either in the form of small membrane vesicles or after cleavage by phospholipase C or D (Meri et al 1996). The circulating pool of sRAGE consists of esRAGE and proteolytically cleaved forms of RAGE (Hudson et al 2005). Intravascular TFPI is present in three different pools: the first pool is in the circulation (80% in complex with lipoproteins and about 5-20% free), the second is found in the cytoplasm of platelets, and the third, and the largest, pool is bound to the endothelium (DelGiudice and White 2009). Heparin and glycosaminoglycans, and probably endothelial damage, induce the release of free TFPI from the vessel wall (Sandset et al 1988).
In this study, the AP patients with OF were found to have PC deficiency and decreased APC generation, increased serum levels of CD59, increased plasma levels of sRAGE and increased plasma levels of free TFPI. It thus seems that our findings in severe AP patients with OF could be explained by endothelial damage caused by AP.

Patients with severe AP develop systemic inflammation, which is considered to play a role in the pathogenesis of MOF. OF mimics the process seen in patients with sepsis: it is characterized by an overwhelming production of mediators of inflammation, activation of the complement system and activation of coagulation. These pathways interact in many ways during systemic inflammation. The key factor linking inflammation and coagulation is TF, which is expressed by monocytes after cytokine stimulation, and exposed to blood after endothelial damage (Esmon 1999; Levi and van der Poll 2008). The induction of coagulation by TF leads to thrombin generation. Thrombin and proinflammatory cytokines then activate platelets (Dahlback 2000), and this further enhances TF expression (Mackman 2004). The function of natural anticoagulants is impaired by proinflammatory mediators, and the ongoing thrombin generation leads to their consumption thus lowering their levels (Esmon 2000; Levi and van der Poll 2008). In experimental studies, cytokines have been found to down-regulate TM and EPCR expression (Esmon 1999). EPCR can be shed from endothelium by thrombin and inflammatory mediators, and it can bind to activated neutrophils, inhibiting their extravasation (Esmon 2004a). TM can bind to and neutralize HMGB1 (Abeyama et al 2005; Esmon 2005b). The complement pathway regulator protein C4Bp binds to PS and diminishes its cofactor effect on PC (Vine 2009). Complement activation generates the MAC, which can expose the phosphatidylserine on the surface of the cells, where it can take part in the initiation and amplification of coagulation (Esmon 2004a). Thrombin activates TAFI, which is an inhibitor of the complement anaphylatoxin C5a (Esmon 2005a).

Naturally, all these interactions between inflammation and coagulation could, at least partly, explain our findings in the patients with severe AP.

3.4.4 Limitations of the study

The main limitation of this study is the relatively small number of patients in the four parts of the study. Therefore, regardless of our interesting findings about the inflammatory and coagulation disturbances in AP patients, and the association between the development of OF and fatal outcome, these findings should be confirmed in further studies with a larger number of patients. In addition to the small patient material, another limiting factor was the lack of sufficient samples during the study period. This was due mostly to the early discharge of the patients from hospital, but also some samples were lost during the storage period.

In the coagulation studies, there may have been some confusion and limitations regarding the patients' medications. Some patients may have used anticoagulants before admission to hospital, or may have received prophylactic medication for thrombosis, or coagulation products during their hospital stay. Some of the patients with alcohol-induced AP may have had nutritional deficiencies or liver failure, which may have influenced their coagulation capacity.

In our coagulation studies we investigated thrombin generation capacity and two of the three endogenous anticoagulant pathways, namely PC and TFPI. However, we did not study AT, which also has a central role in the coagulation and inflammation processes. In these studies we could also have used the DIC score, which would have given us more information about the patients' coagulation status.

In our studies on inflammation markers, we measured the levels of HMGB1, which has been widely investigated in sepsis. This was logical, because the SIRS in AP resembles sepsis. RAGE is a receptor for HMGB1 and has a soluble form, sRAGE. High sRAGE levels have also been found in sepsis and acute lung failure, but chronic diseases have been the most frequent target of investigation. Because acute lung failure is the commonest OF in AP, we decided to study the sRAGE levels together with the HMGB1 levels. We found elevated sRAGE levels in AP patients with OF, and the levels were also higher in non-survivors than in survivors. However, only little is known about the functioning of sRAGEs in patients. So, despite our interesting findings, it is difficult to determine their clinical importance. On the other hand, it is known that CD59 deficiency in humans causes a disease called paroxysmal nocturnal hemoglobinuria, which is characterized by hemolytic anemia and thrombosis.

Similarly, mice deficient in AT, TFPI and PC die already in the uterus during embryonic development.

3.4.5 Clinical and future aspects

Despite the increasing knowledge of the pathogenesis of AP (mainly from animal studies) and OF, the treatment of AP has advanced only little, and is currently still mainly supportive. It is known that in AP, the development of OF is most important factor leading to morbidity and mortality. Especially if OF is present already at admission to hospital (early OF) and is persistent (lasting >48 h) the mortality rate is high, and in over half of the patients, OF progresses to MOF. Thus, early identification of patients who are likely to develop OF is important, so that aggressive supportive treatment could be started in the intensive care unit. It is noteworthy that the 'time window' for early recognition of these patients and commencement of the possible treatment is very short, probably less than 24 h.

In this study, we tried to find inflammatory and coagulation markers that would predict the development of OF and fatal outcome in AP. All of the OF patients studied had acute lung failure, necessitating mechanical ventilation and/or renal failure necessitating hemodialysis. All of the patients with OF also had persistent OF. We found defects in the PC pathway, which were associated with the development of OF. Similarly, we found that high levels of free TFPI, sRAGE and CD59 were associated with the development of OF, and that high CD59 levels predicted the development of OF. The levels of free TFPI combined with a 'flat curve' in the thrombogram were also predictive for fatal outcome in AP. These findings might reflect endothelial damage caused by systemic activation of inflammation and coagulation in AP. However, before these markers could be considered as predictive for OF in clinical use, these findings should be confirmed in studies with a larger number of AP patients. Also, the used analytical methods are time-consuming or are not currently used in clinical practice, and thus are not feasible tools in emergency medicine.

Could these findings be useful in therapeutic approaches? In experimental studies, administration of recombinant sRAGE and soluble CD59 has been shown to suppress the inflammatory response. The use of anti-HMGB1 neutralizing antibody was found to decrease histological alterations in pancreas in an experimental AP study. In a clinical study, recombinant APC has been shown to reduce mortality in sepsis patients. Similarly, administration of recombinant TFPI has been studied with controversial results in sepsis

patients. These findings should nevertheless be confirmed in large clinical trials with AP patients.

In the future, it would seem profitable to study more closely the mechanisms behind the endothelial damage in SIRS and AP. By studying the linking factors between the coagulation and inflammatory cascades, it might be possible to find therapeutic agents and to start clinical trials. Currently, the natural anticoagulant pathways seem to be promising candidates for the clinical studies, because they express both anticoagulant and anti-inflammatory effects. Furthermore, the thrombogram method might offer a useful tool for investigating in more detail the kinetics of coagulation, and the coagulation disturbances in AP.

3.5 Conclusions

1. PC deficiency and decreased APC generation in severe AP probably contribute to compromised anticoagulant and anti-inflammatory defence. A new coagulation disturbance (i.e., TF-initiated thrombin generation delayed by TFPI release) in AP was characterized.

2. Increased serum levels of complement regulator protein CD59, and increased plasma levels of sRAGE were found in patients with severe AP and OF. There was no significant difference in the HMGB1 levels between the severity groups of AP patients. Thus, CD59 and sRAGE may play a role in the pathogenesis of inflammation in these patients.

3. The defects in the PC pathway and TFPI-release-induced delayed thrombin generation might be associated with the development of OF in AP. Also, the increased levels of CD59 and sRAGE seem to be associated with the development of OF in AP. The ‘flat curve’ in CAT combined with free TFPI (cutoff 25 ng/ml) seem to be predictive of fatal outcome. Also, the high sRAGE level might be predictive of fatal outcome.

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